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IDENTIFICATION OF SMALL MOLECULE REGULATORS OF CILIOGENESIS: A NEW APPROACH TO CANCER DRUG DISCOVERY AND THERAPY

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Abbreviations:

AC	Adenylate cyclase
ADPKD	Autosomal dominant polycystic kidney disease
AHI1	Abelson helper integration site 1
ANOVA	Analysis of variance
APC	Adenomatous polyposis coli, Anaphase promoting complex
ARL	ADP-ribosylation factor (ARF)-like
ARPKD	Autosomal recessive polycystic kidney disease
ATCC	American type culture collection
ATP	Adenosine triphosphate
AURKA	Aurora kinase A
BBS	Bardet-Biedl syndrome
BCC	Basal cell carcinoma
BHD	Birt-Hogg-Dube syndrome
BrdU	Bromodeoxyuridine
BSA	Bovine serum albumin
CALK	Chlamydomonas aurora-like protein kinase
cAMP	Cyclic adenosine monophosphate
CBF	Ciliary beat frequency
ccRCC	Clear cell renal cell carcinoma
cdc20	Cell division cycle 20
CDK	Cyclin-dependent kinases
Cep	Centrosomal protein
cGMP	Cyclic guanosine monophosphate
CISTIM	Center for Innovation and Stimulation of Drug Discovery
CK1	Casein kinase 1
CNS	Central nervous system
COX	Cyclooxygenase
CP	Centrosomal protein
CRL3	Cholesterol esterase
DAMP	Damage-associated molecular pattern
DAPI	4',6-Diamidino-2-Phenylindole, Dihydrochloride
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DNAH	Dynein axonemal heavy chain
DPBS	Dulbecco's phosphate-buffered saline
EGFR	Epidermal growth factor receptor
ER	Endoplasmic reticulum
FACS	Fluorescence-activated cell sorting
FAP	Familial adenomatous polyposis
FBS	Fetal bovine serum
FBW7	F-box and WD repeat domain-containing 7
FCS	Fetal calf serum
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
FLCN	Folliculin
FNIP	Folliculin interacting proteins
FOXJ1	Forkhead box J1
Gli	Glioma
GPCR	G protein coupled receptors
GPS	Global positioning system
GSK3	Glycogen synthase kinase 3
HDAC6	Histone deacetylase 6
HEF1	Human enhancer of filamentation 1
hESC	Human embryonic stem cells
HSP	Heat shock protein
IFT	Intra flagellar transport
IGF-1R	Insulin-like growth factor 1 receptor
INPP5E	Inositol polyphosphate-5-phosphatase E
JBTS	Joubert syndrome
KCTD17	Potassium channel tetramerization domain containing 17

KIF	Kinesin-like protein
LEF	Lymphocyte enhancer factor
MAPK	Mitogen-activated protein kinases
MARK4	MAP/Microtubule affinity-regulating kinase 4
MDCK	Madin-Darby canine kidney
MEF	Mouse embryonic fibroblasts
MEK	Melanoma knowledgebase
MIM	Missing-in-metastasis
MKS	Meckel syndrome
MST1R	Macrophage stimulating 1 receptor
MTOC	Microtubule-organizing center
mTOR	Mechanistic target of rapamycin
Nde1	NudE neurodevelopment protein 1
NEDD9	Neural precursor cell expressed developmentally down-regulated protein 9
NEK	NIMA-related kinase
NF-κB	Nuclear factor-κB
NPHP	Nephronophthisis
OFDS	Oral-facial-digital syndrome
ORPK	Oak ridge polycystic kidney
OSN	Olfactory sensory neuron
PanIN	Pancreatic intraepithelial neoplasia
PC	Polycystin
PCD	Primary ciliary dyskinesia
PCP	Planar cell polarity
PDAC	Pancreatic ductal adenocarcinoma
PDGFR	Platelet-derived growth factor receptor
PKD	Polycystic kidney disease
PKHD	polycystic kidney and hepatic disease
POMC	Pro-opiomelanocortin
PTCH1	Patched 1
PTM	Post-translational modification
RFX	Regulatory factor X
RPGRIP1L	Retinitis Pigmentosa GTPase Regulator Interacting Protein 1-like
RPMI	Roswell Park Memorial Institute
SAR	Structure-activity relationship
SEPT	Septin
Shh	Sonic hedgehog
siRNA	Small interfering RNA
SREBP1	Sterol regulatory element-binding protein 1
SSTR	Somatostatin receptor
SSX2IP	Synovial sarcoma X breakpoint 2 interacting protein
SUFU	Suppressor of fused homolog
TAZ	Transcriptional coactivator with PDZ-binding motif
TCF	T cell factor
TCTN	Tectonic proteins
TGF	Transforming growth factor
TGN	Trans-golgi network
TMEM	Transmembrane protein
TRP	Transient receptor potential
TSC	Tuberous sclerosis complex
TTBK2	Tau tubulin kinase 2
TTC21B	Tetratricopeptide repeat protein 21B
TZ	Transition zone
UPS	Ubiquitin proteasome system
VHL	Von Hippel-Lindau
ZNF	Zinc finger protein

CHAPTER 1

Introduction

1.1. CILIA

Cilia are microscopic hair-like organelles present on the surface of most mammalian cells. These microtubule-based structures form flexible extensions of the cell membrane and play a vital role in the development, and normal functioning of humans and animals. Cells may possess multiple cilia or a single cilium on their surface. Dysfunction of the cilium is associated with many different pathologies.

1.1.1. Types of Cilia

There are several kinds of cilia that play different roles in biological processes. They can be broadly classified into three categories - motile, primary and nodal cilia.

1.1.1.1. Motile cilia are generally found on airway epithelia, cells lining the oviduct and ependymal cells in the brain [1]. Usually, these cells are multi-ciliated. These kind of cilia are mechanical in function and move in an organized wave-like pattern, which is necessary for fluid flow such as clearance of mucus in the lungs, movement of oocytes, movement of cerebrospinal fluid in the brain, etc. [2, 3].

1.1.1.2. Primary cilia are immotile and exist as monocilia on most cells of the human body [4]. They play a sensory role and enable the cells to communicate and interact with the surrounding environment by capturing and transducing various extracellular signals like chemical, mechanical and visual stimuli [5]. Recent findings show that primary cilia play an important role in a number of signaling cascades including hedgehog, wnt, and PDGFR-α [6-9].

1.1.1.3. Nodal cilia are found on the embryonic node during development. These are a type of primary cilia that have the ability to move with rotational motion along a vertical axis in a propeller-like fashion. This motion is thought to aid the movement of morphogens in the developing tissue. Nodal cilia have been implicated in the specification of left-right asymmetry of the body axis [10-12].

1.1.2. Evolution of cilia: sensory or motile cilia, which came first?

Several interesting hypotheses were formulated in the past to explain the evolutionary origin of cilium. Being ancient organelles present in almost all eukaryotes, it is very likely that they were present on the last common eukaryotic ancestor. It is believed that the cilium has evolved by adaptation of existing structures that are involved in microtubule organization and membrane trafficking [13-15]. In view of the seemingly

disparate functions of sensory and motile cilia, attempts have been made recently to understand the evolutionary history. Despite the likelihood that ancestral cilium did have sensory functions, it is not yet clear whether this sensory function emerged before, or after, the acquisition of motile function. Peering back in time, it appears that the precursor of ancestral cilium might have been a sensory patch on the surface of a polarized cell that ultimately extrapolated into an antenna-like structure that was efficient in further concentrating receptors and signaling components (Figure 1.1). The apparatus necessary for motility is likely to have developed later with the appearance of a proto-cilium [15, 16]. It is believed that the nine-fold symmetry of the basal body and axoneme evolved to confer motility to the cilium. The ancestral cilium, was thus endowed with both sensory and motile functions. Only a few eukaryotic lineages derived from the ancestral eukaryote have lost cilia altogether, whereas motility has been lost in a diverse set of metazoan cell-types.

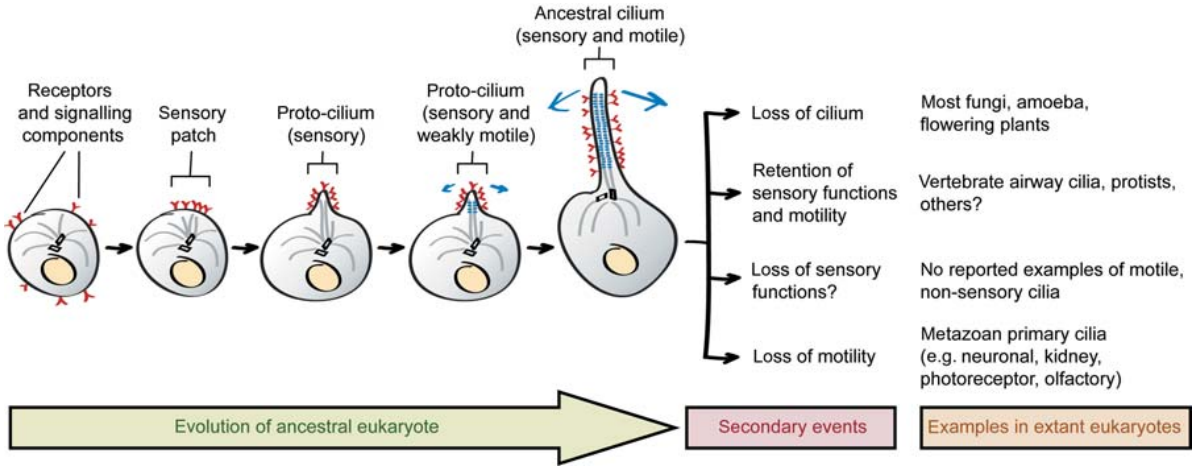

FIGURE 1.1. Possible evolutionary path explaining the emergence of the ancestral ciliated eukaryote, and subsequent cilium-centric events leading to extant organisms. Cells are shown with a microtubule organizing centre from which microtubules (gray) emanate, or in the ancestral eukaryote as a basal body from which a ciliary axoneme emanates. The microtubule-dependent clustering of sensory molecules (red) was likely the first step before the appearance of a proto-cilium with an even more concentrated sensory-signalling environment. The apparatus used to confer motility, shown as blue decorations on the microtubules/axoneme, likely evolved early in the proto-cilium. Both ciliary motility and sensory functions undoubtedly conferred a strong selective advantage to the ancestral eukaryote, such that all extant eukaryotes are derived from it. Only a few eukaryotic lineages have lost cilia altogether, whereas motility has been lost in numerous metazoan cell types. (Figure taken from [16]).

1.1.3. Structure of the cilium

The cilium is a highly conserved organelle that consists of a microtubule-based axoneme, which is surrounded by a specialized membrane called the ciliary membrane. The axoneme emerges from a basal body, which is essentially a modified centriole. The inner space of the cilium is separated from the cytosol by the transition zone. Figure1.2 represents the basic structure of a primary cilium.

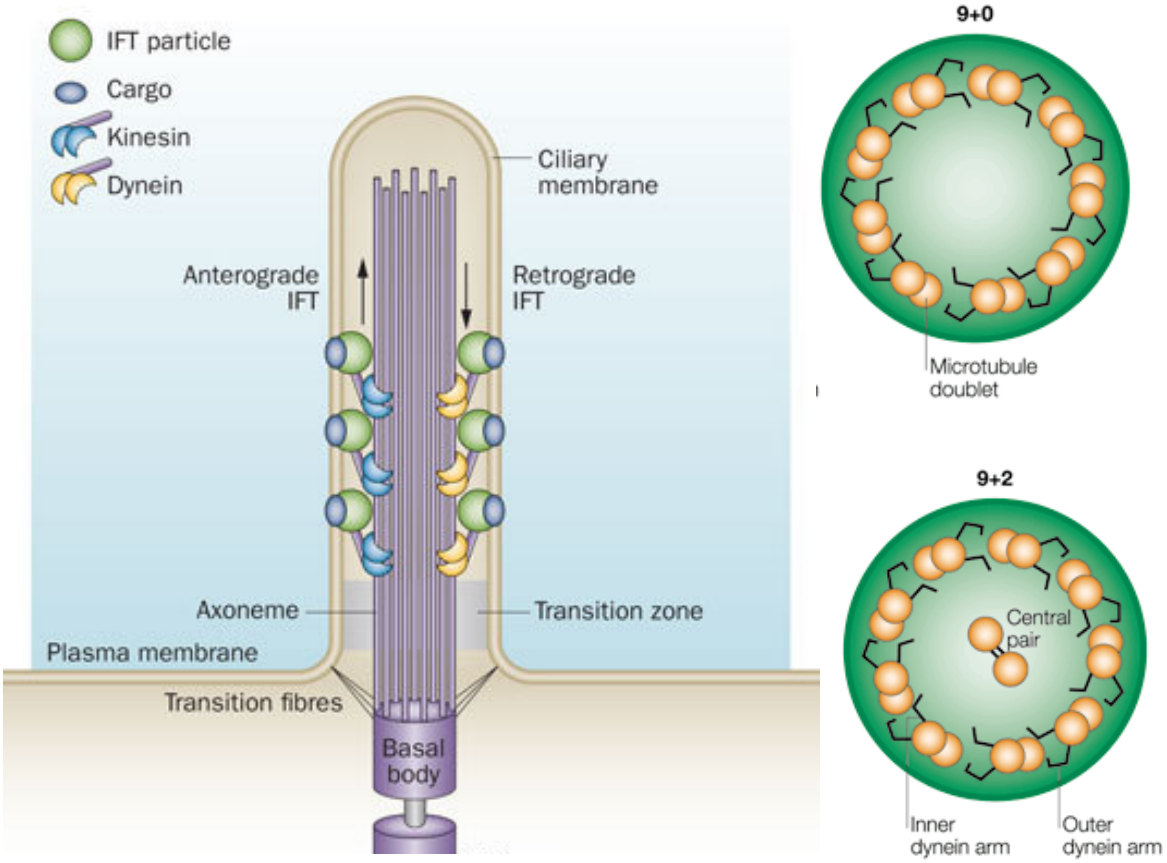

FIGURE 1.2. Structure of the cilium: The cilium is a hair-like structure that protrudes from the cell surface. Microtubules form the core structure of the cilium, the axoneme, which is covered by a ciliary membrane. The basal body anchors the axoneme to the rest of the cell. Near the base of the cilium is the transition zone, which allows selective access to only those proteins that are destined to the cilium. Protein cargo is transported up and down the cilium via anterograde and retrograde IFT mediated by kinesin and dynein motor proteins, respectively, which travel along the axoneme. Right hand side of the figure shows two distinct types of cilium. '9+0' cilia, also known as primary cilia or monocilia, contain a ring of nine peripheral doublets of microtubules but lack the central pair, whereas '9+2' cilia, also known as motile cilia, contain the central pair. (Figure taken from [115] & [182]).

1.1.3.1. Axoneme: The ciliary axoneme contains a cytoskeleton of microtubule doublets arranged in the form a ring. The ring may have a 9+0 or 9+2 arrangement of microtubules (Figure 1.2). Motile cilia display the 9+2 arrangement in which nine microtubule doublets surround a central pair of microtubule singlets. Primary cilia have a 9+0 arrangement, which lacks the central pair of microtubules. Historically, the 9+0 configuration is associated with cilia having sensory function whereas the 9+2 configuration is associated with motility. Several studies suggest that post-translational modifications (PTMs) of axonemal components are important for the assembly, stability and function of the cilium. Enzymes that interfere with PTMs of axonemal components are known to either promote disassembly or cause a delay in the assembly of the primary cilium [17-19]. Tubulin, the main component of microtubules has been shown

to be acetylated [20], detyrosinated [21], polyglutamylated [22] and polyglycylated [23]. It is possible that such PTMs are required for the binding of specific proteins that interact with microtubules to control critical steps in the cilium building process [24].

1.1.3.2. Basal Body: The basal body connects the axoneme to the rest of the cell. It functions as a microtubule-organizing center (MTOC), which anchors the microtubules from which the axoneme is templated. The basal body is essentially a modified centriole that differs from a centriole by the addition of three accessory structures namely, striatal rootlets [25], basal feet and transition fibers [26]. The rootlets extend from the basal body into the cytoplasm [27] and are essential for the structural integrity and long-term survival of the primary cilium [28]. Basal feet emerge laterally from the side of basal body and stabilize the microtubules [29]. Under the regulation of planar cell polarity (PCP) proteins, the basal feet also control the polarization of the cilium [30]. The distal appendages of the mother centriole give rise to transition fibres, whose primary role is to anchor the basal body to the ciliary membrane at the ciliary pocket. However, recent studies have shown that transition fibres are also required for docking intraflagellar transport (IFT) particles [31]. Other proteins such as septin 2 (SEPT2) are also known to be targeted to transition fibres [32]. Overall, the basal body acts as an interface between the primary cilium and the cell body. It also provides a docking area for a large number of proteins, which can have a positive or negative effect on cilia formation and maintenance [33]. The basal body therefore, plays a key role in the assembly and disassembly of the primary cilium.

1.1.3.3. Ciliary Membrane: The ciliary cytoskeleton is enveloped by a ciliary membrane, which is continuous with the surrounding plasma membrane but distinct in overall composition. In comparison to the plasma membrane, the ciliary membrane is enriched in sterols, sphingolipids and glycolipids. It is more complex and specialized than the plasma membrane [34] also in the sense that it has a high concentration of specific receptor proteins and channels on its surface, which are vital for the detection of particular environmental signals [35]. A large number of membrane receptors and proteins including morphogenic receptors like Patched1 (Ptc1), mechanosensory receptors like PKD1 and PKD2, receptors for growth factors, calcium channels, polycystins etc. have been shown to be localized to the ciliary membrane [34]. In most cases, the functionality of these receptors is dependent on their localization to the ciliary

membrane [7, 9, 36]. Unraveling the molecular mechanisms that choreograph the movement of these receptors and channels to the ciliary membrane is critical to understanding the role of cilia-generated signaling in health and disease. Discovery of small molecules that can inhibit or induce the targeting of clinically significant proteins to the ciliary membrane is gaining ground as the next focus of translational research involving the primary cilium.

1.1.3.4. Transition Zone: At the base of ciliary axoneme is a region called transition zone (TZ), where the triplet microtubule structure of the basal body converts into the axonemal doublet microtubule structure. The TZ contains Y-shaped links, which connect the outer microtubule doublets of the axoneme to the ciliary membrane [37, 38]. The TZ allows only a subset of cellular proteins to enter the cilium. Thus, it forms a selective functional barrier near the base of the cilium that separates the ciliary membrane from rest of the cell membrane [34]. This diffusion barrier is composed of two distinct regions called the ciliary necklace and the ciliary pocket. Visualization by electron microscopy revealed that the ciliary necklace is composed of several parallel strands of intramembrane particles that connect the proximal end of the ciliary membrane to the basal body [39]. It acts as a nuclear membrane and controls the entry or exit of proteins to or from the ciliary axoneme. Ciliary pocket appears as an invagination of the plasma membrane at the ciliary base [40]. It is considered as a rate-limiting structure for the trafficking of cilia-related proteins [41]. All proteins that enter the cilium must pass through this diffusion barrier, which results in the compartmentalization of ciliary signaling proteins [32, 37], a vital requirement for the sensory function of the primary cilium. The mechanism of action of TZ-associated proteins is largely unclear although several of them are known to contain membrane-associated domains, which is consistent with their role as a diffusion barrier [37, 42, 43].

1.1.4. Formation of the cilium

Cilia are post-mitotic structures that are typically formed during the G0 phase of the cell cycle. They arise only from the mother centriole, as the daughter centriole is still immature [44]. The mother centriole can be distinguished from the daughter centriole by the presence of distinct appendages. The currently accepted model of cilium formation is derived from electron microscopic analysis of organ cultures and fibroblasts [45]. The steps proposed in ciliogenesis are illustrated in Figure 1.3.

Formation of the cilium begins when a Golgi-derived vesicle docks near the distal appendages of the mother centriole migrating towards the apical plasma membrane [46]. The docking is mediated by transition fibres, which are accessory structures

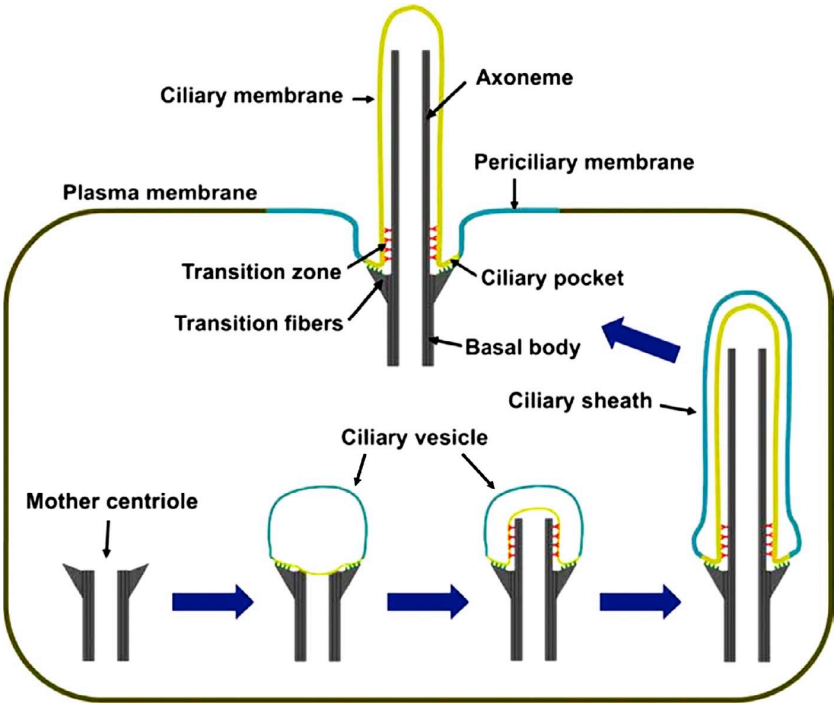

FIGURE 1.3. Ciliogenesis: Cilium formation starts when a mother centriole contacts a ciliary vesicle. Axonemes elongate at their tips and so are constructed from proximal to distal, with the most proximal region giving rise to the transition zone. The ciliary vesicle grows with the axoneme and gives rise to the ciliary sheath, whose fusion with the plasma membrane externalizes the cilium and transforms the outer sheath into the periciliary membrane. (figure taken from [49]).

derived from the distal appendages of the mother centriole to offer structural support [31, 47]. The mother centriole converts into a basal body from which a bud emerges to bend the membrane of the vesicle [48]. The bud elongates from the tip as newly assembled microtubule doublets emerge from the basal body to allow the formation of an axoneme [49]. The base of the bud becomes the transition zone where the nine microtubule triplets become doublets and each doublet is connected to the ciliary membrane through Y-links to form a ciliary necklace [50, 51]. More vesicles fuse with the migrating vesicle-basal body complex, thereby providing membrane material to form a sheath around the elongating axonemal shaft. Finally, the growing axoneme reaches the cell surface, allowing the membrane sheath to fuse with the plasma membrane, exposing the cilium to extracellular space [34, 52]. The newly formed axoneme elongates further till it reaches a stable length [53].

1.1.5. Regulation of the cilium

1.1.5.1. Assembly of the cilium: Since the cilium is devoid of ribosomes and protein biosynthetic machinery, all components required for cilia assembly are synthesized in the cell body and then transported to the cilium by a unique trafficking mechanism called Intraflagellar Transport (IFT) [54]. It is responsible for the maintenance of ciliary structure and function. The IFT machinery consists of at least two molecular motors and large protein complexes called IFT particles. Microtubule motor protein Kinesin 2 is directed towards the plus-end of the cilium (base-to-tip) and is essential for the transport of the protein cargo towards the ciliary tip (anterograde transport), whereas a cytoplasmic dyenin2 carries the axonemal turn-over products towards the minus-end (tip-to-base) direction back to the cell body for recycling (retrograde transport) [55]. Thus, IFT particles resemble constantly moving molecular trucks that run along the microtubule doublets in a closed loop within the ciliary membrane [56] (Figure 1.4). Addition of new material to the distal tip continues even after the cilium reaches its final length. The balance between assembly and continuous turn-over creates a steady state which maintains a fixed length [57]. However, it is still unclear how anterograde kinesin and retrograde dynein motors are switched on or off when the IFT particles reach the tip. The IFT motility process is evolutionarily conserved in ciliated eukaryotes. The IFT particles consist of more than 17 highly conserved proteins, which are organized in two different complexes [58]. Complex A is involved in the trafficking of retrograde cargo whereas Complex B is responsible for the transport of anterograde cargo [59]. IFT-B complex is composed of 14 proteins important for the biogenesis and maintenance of cilium. Loss of any component of the IFT-B complex leads to the disruption of ciliary assembly [52, 60]. This observation is consistent with the notion that IFT-B complex is involved in anterograde transport responsible for the building up of cilium. Knockdown or knockout of several components of IFT-B complex like IFT88 and IFT20 can thus be used as a strategy to suppress ciliogenesis or ciliary function [61]. The IFT-A complex consists of 6 proteins which are not essential for cilium assembly [62]. However, mutations in IFT-A proteins result in short, stumpy cilia with abnormal bulges at the tip that are filled with IFT proteins [52, 63]. It has been observed that IFT particles accumulate at the transition

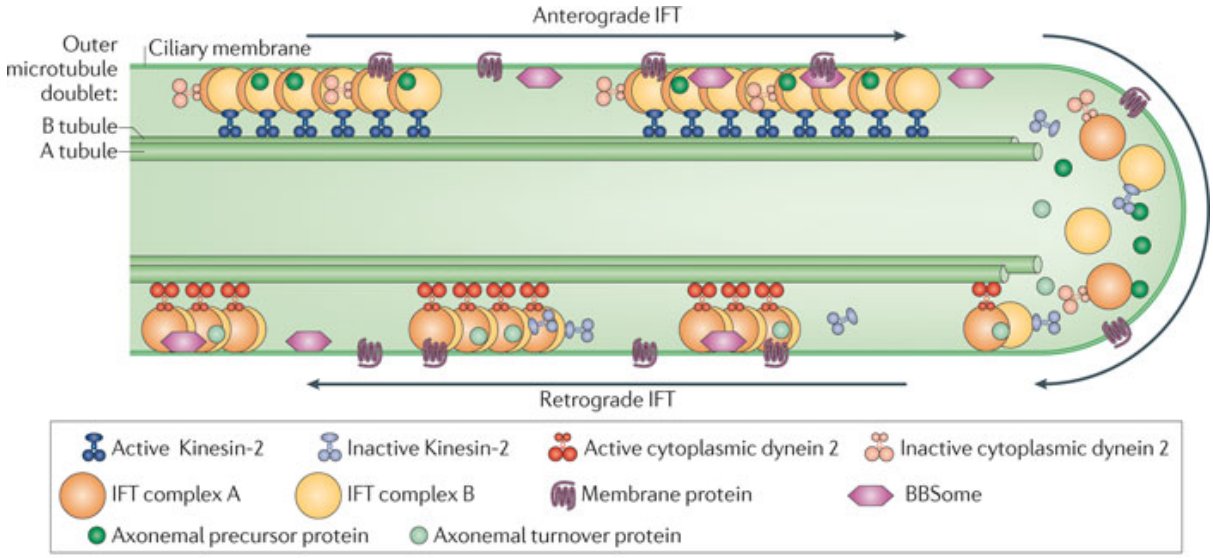

FIGURE 1.4. The Intraflagellar transport machinery: The anterograde intraflagellar transport (IFT) motor, heterotrimeric Kinesin-2, transports IFT complexes A and B, axonemal proteins and cytoplasmic dynein 2 (previously known as cytoplasmic dynein 1b) to the tip of cilium. During this anterograde motion, Kinesin-2 is active and the retrograde motor, cytoplasmic dynein 2, is somehow kept inactive to allow smooth processive anterograde movement. At the tip of cilium, anterograde IFT trains release axonemal proteins and rearrange their conformation for retrograde IFT. Cytoplasmic dynein 2 is activated and transports retrograde IFT trains to the cell body. Subsets of IFT trains are involved in transporting membrane proteins and the BBSome (a complex comprised of at least seven Bardet–Biedl syndrome proteins). (Figure taken from [53]).

fibers, indicating that docking at the transition zone might be a critical regulatory step for the transport of proteins into and out of the cilium [31]. Interestingly, several recent studies have shown that FOXJ1 and RFX family of transcription factors that are involved in cancer, are also involved in the expression of cilia genes associated with intraflagellar transport [64, 65].

1.1.5.2. Disassembly of the cilium: For a cell to proceed into mitosis, the cilium has to be dismantled to free the mother centriole required for cell division. The removal of cilium can occur by two different mechanisms. One of them is by a process called deciliation in which the cell sheds the cilium by katanin-mediated cleavage of the complete cilium from the basal body and transition zone [66]. The other mechanism is characterized by active resorption of cilium as a result of axonemal disassembly starting from the ciliary tip. It is accompanied by an increase in the number of empty IFT particles moving in the anterograde direction, while IFT particles moving in the retrograde direction continually return ciliary proteins to the cell body [67]. The cessation of axonemal subunit delivery to the distal tip, coupled with continuous transport of disassembly products back to the cell body, ultimately leads to the

resorption of cilium. Aurora A Kinase, a regulator of mitotic entry, is known to initiate resorption of cilia [17]. Interaction of Aurora A Kinase with HEF1 at the basal body results in phosphorylation and consequent activation of HDAC6, which promotes ciliary disassembly by reducing microtubule stability [17]. Recently, a protein called Pitchfork (Pifo) was found to accumulate at the basal body, where it activates Aurora A Kinase to promote the disassembly of cilium [68]. Another protein that promotes protein disassembly is Tctex-1, a light chain subunit of cytoplasmic dynein, which is recruited to the transition zone before S-phase. It controls ciliary resorption when uncoupled from the dynein complex [69].

The assembly and disassembly of the cilium is tightly coupled to the cell cycle. Figure 1.5 depicts the various stages of ciliary assembly and disassembly with respect to the progression of cell cycle.

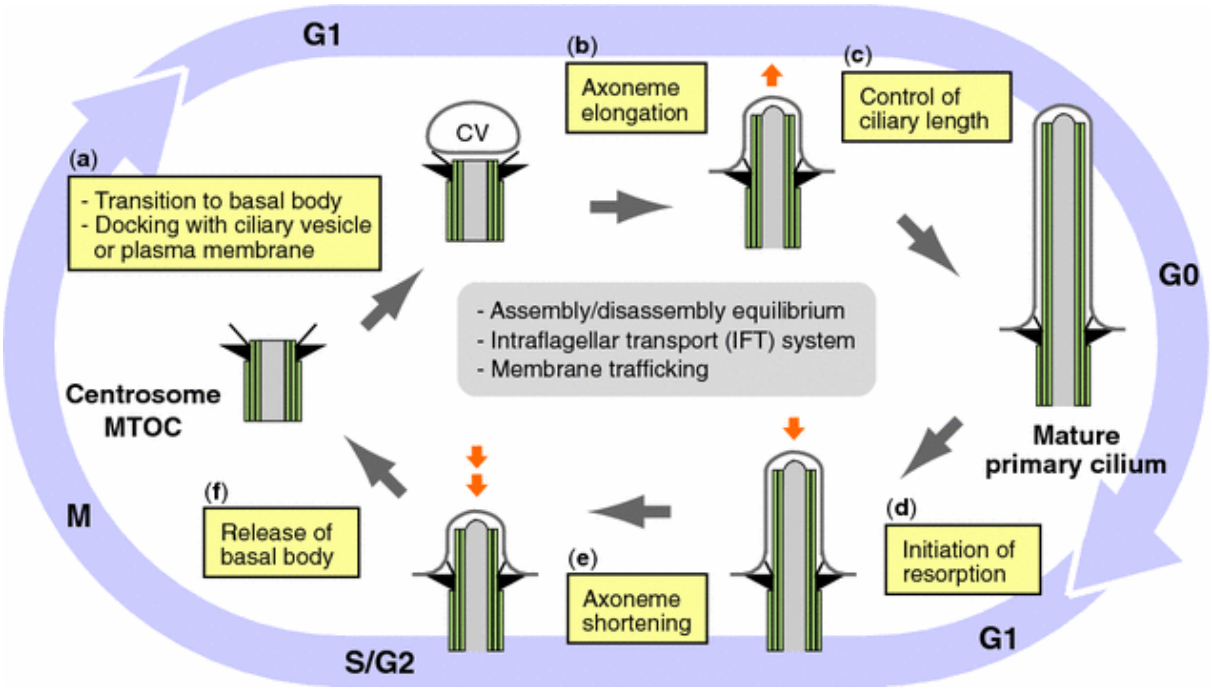

FIGURE 1.5. Ciliogenesis cycle and cell cycle: Multiple steps (**a**–**f**) of primary cilia formation in ciliogenesis cycle are shown in relation to cell cycle. (Figure taken from [80]).

1.1.5.3. Regulation of cilium length: Ciliary length is an important parameter as it can critically affect the function of cilia, especially of cilia that are involved in the generation of specific fluid movements. It is important for cells to have cilia of normal length, as either shortened or elongated cilia can lead to developmental defects or diseases. It has been proposed that ciliary length depends on the balance of assembly and disassembly at the ciliary tip [57]. An inverse relationship exists between the efficiency of IFT and

ciliary length. As length of the cilium increases, the frequency of cargo delivery decreases due to the fact that it has to traverse a larger distance before reaching the tip. This results in a 'Balance-point' model in which IFT represents the main control mechanism for the regulation of cilium length through changes in the IFT machinery, such as cargo selection, IFT particle size, trafficking speed and frequency of trafficking. This IFT control also occurs at the transition zone [70] which houses a number of structural proteins like NPHP8, Tmem67, Cc2d2, Tctn1 and Tctn2 that are essential for the elongation of cilia [71, 72]. These proteins modulate ciliary length by possibly regulating the recruitment of ciliary proteins from the cytosol to the cilium.

Recent studies propose that regulation of the cilium length is achieved by the recruitment of positive and negative regulators of ciliogenesis at early stages of basal body conversion [73]. The basal body acquires positive regulators of ciliogenesis and removes the negative regulators before docking to the plasma membrane to begin axonemal extension. Several proteins have been identified as key regulators in this process. CP110, a negative regulator of cilia formation, interacts with Cep290 to restrain ciliogenesis in dividing cells [74]. It has been shown that proteins tau tubulin kinase 2 (TTBK2) and microtubule- associated protein/microtubule affinity regulating kinase 4 (MARK4) initiate ciliogenesis by dislocating CP110 from the mother centriole [75]. Besides CP110, the protein trichoplein acts as a negative regulator of ciliogenesis by activating Aurora-A kinase in the cycling cells [76]. A recent study shows that trichoplein is degraded at an early stage of ciliogenesis by CRL3-KCTD17 complex of the Ubiquitin proteasome system (UPS) [77]. Another study has demonstrated that oral-facial digital syndrome 1 (ofd-1) protein is required for primary cilia formation [78]. It exerts a cilia-suppressive role when present in the centriolar satellites. Induction of selective autophagy by serum starvation removes ofd1 from centriolar satellites to centrioles resulting in the formation of longer cilia [79].

Emerging evidence has revealed several mechanisms that are involved in the control of ciliary length linked to the cell cycle (Figure 1.6) [80] (see also 1.1.6). Nde1, which has been shown to suppress ciliogenesis by affecting cell cycle re-entry [81], controls ciliary length through phosphorylation by CDK5 that primes Nde1 for FBW7 E3 ubiquitin ligase-mediated degradation by the Ubiquitin-proteasome system [82]. Another mechanism involves Anaphase Promoting Complex (APC), which interacts with cdc20 to maintain ciliary length, and is important for the timely resorption of cilium [83]. The APC-cdc20 complex controls the length of cilium by regulating the stability of

microtubules in the axoneme by targeting Nek1 for proteolysis [83]. Some studies show that GTPase Arl13b localizes to the cilium and regulates ciliary length [84]. It has been proposed that Arl13b regulates the length of cilium by recruiting proteins of the BBS complex to the cilium [85]. Some pharmacological compounds like Lithium and Forskolin, an activator of adenylate cyclase (AC) are known to elongate cilia by altering the intracellular levels of second messengers like calcium and cAMP followed by activation of protein kinase A [86-88]. Ciliary length increases with lowering of Ca^{2+} and elevation of cAMP levels. Under fluid flow conditions, the cilium length decreases as intracellular Ca^{2+} levels rise and cAMP levels decrease [87].

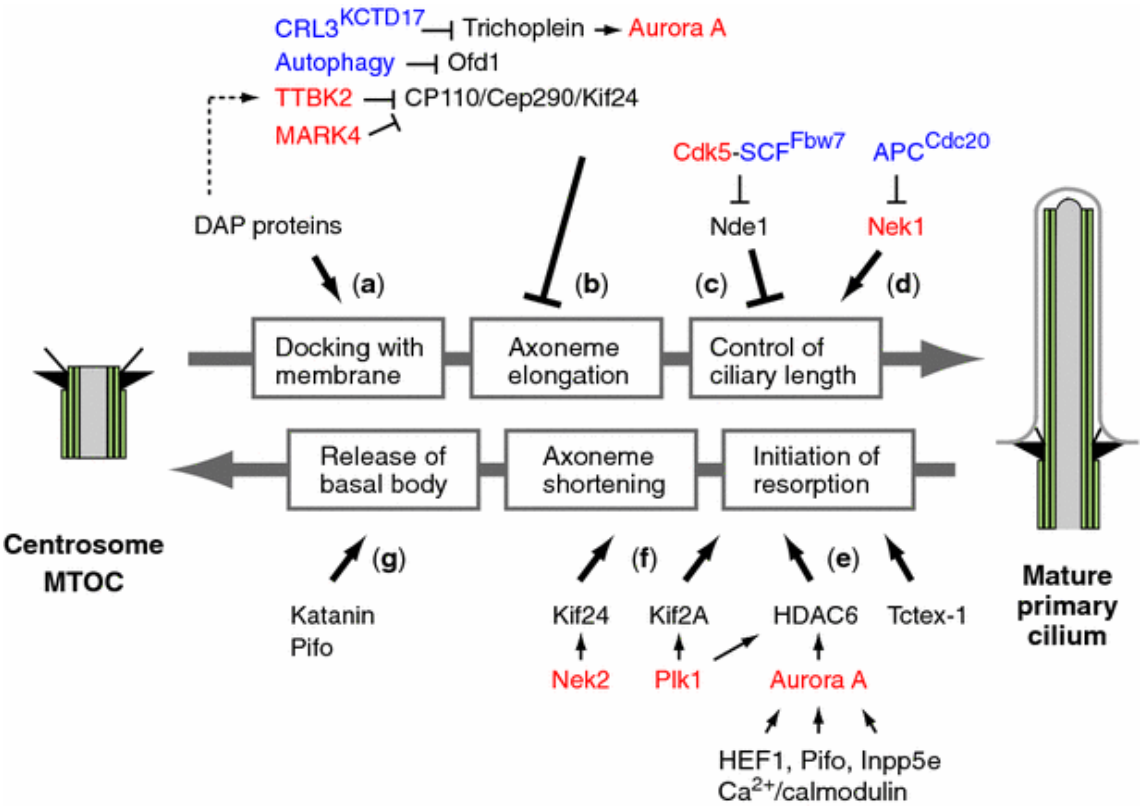

FIGURE 1.6. Molecular mechanisms linking ciliary length and cell cycle regulation: Recent findings to uncover the molecular link between the individual step (**a–g**) of ciliogenesis and cell cycle are depicted. *Red letters* indicate kinases and *blue letters* indicate molecules related to ubiquitin–proteasome pathway or autophagy. (Figure taken from [80]).

This negative feedback response is required to decrease the mechanosensitivity of cilia to fluid shear, which is necessary for the normal functioning of cells involved in the sensing of fluid movements [89]. Recent studies show that Rer1p, a protein quality control receptor is involved in the regulation of ciliary length and function by increasing the assembly and activity of gamma secretase complex, which consequently enhances Notch signaling and reduces Foxj1a expression [90].

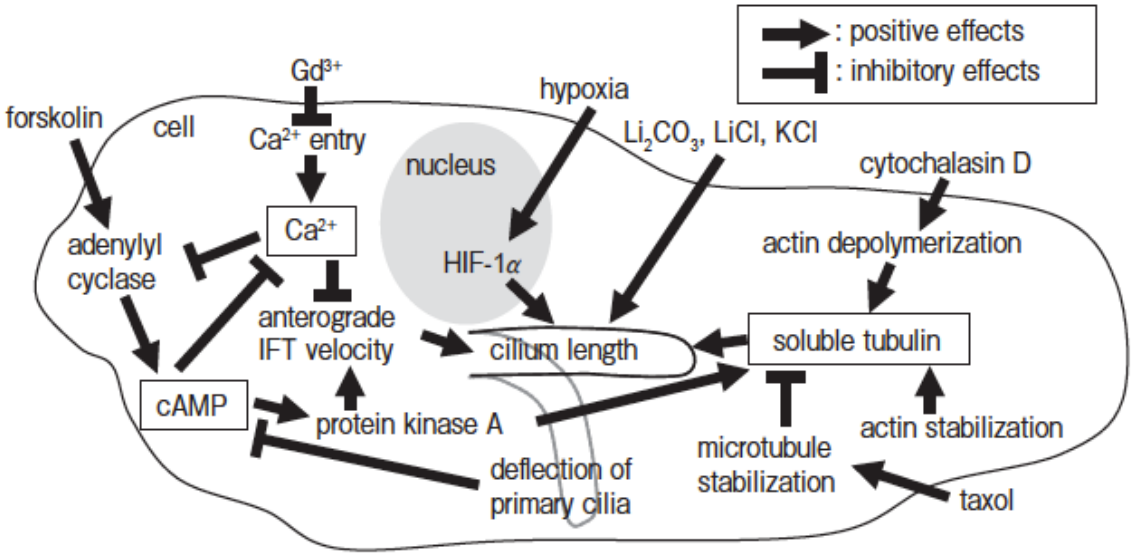

FIGURE 1.7. Proposed pathways by which environmental and pharmacological factors influence primary cilium length: Treatment with Gd3+ or forskolin evokes the elongation of primary cilia via crosstalk between a decrease in the intracellular Ca2+ and an increase in the cyclic AMP (cAMP) level with subsequent protein kinase A activation. The deflection of primary cilia downregulates the intracellular cAMP level, resulting in primary cilia shortening and a subsequent decrease in the mechanosensitivity of primary cilia. Increased velocity of anterograde IFT is thought to underlie the cAMP/Ca2+-mediated elongation of primary cilia. Treatment with cytochalasin D or taxol affects the dynamics of actin filaments and microtubules, respectively, leading to cilia length alteration through modulating the levels of soluble tubulin available for primary cilia extension. Forskolin-induced elongation of primary cilia is attenuated by treatment with taxol, where the effects on cilia length correlate with the levels of soluble tubulin. The mechanism by which treatment with Li+ or K+ elongates primary cilia is uncertain but might be related to certain actions common to monovalent cations. Among the consequences of renal injury, hypoxia is likely to contribute to the elongation of renal primary cilia via hypoxia-inducible factor alpha (HIF-1α). (Figure taken from [88]).

Also several environmental and pharmacological factors are known to influence the length of cilium (figure 1.7). For example, pharmacological regulation of actin and microtubule dynamics for instance by treating cells with cytochalasin D, an inhibitor of actin polymerization affects primary cilium length [91, 92]. Environmental insults like renal injury are also known to trigger alterations in ciliary length. It has been reported that primary cilia length increases in injured kidneys of mice and in human renal transplants affected with acute tubular necrosis [93-95]. It is considered that the elongation of primary cilia in kidneys following renal injury leads to an increase in their sensory capacity, which might promote epithelial differentiation for renal repair.

1.1.5.4. Selective access and targeting of proteins to the cilium: The cilium is a specialized cell surface structure whose function is critically dependent on its composition. Many important proteins are highly enriched in the cilium. To concentrate

these proteins in the ciliary compartment, efficient mechanisms are necessary to restrict their movement in and out of the compartment. This control of entry and exit is tightly regulated at the ciliary base, which is the only region of the cilium not surrounded by a membrane. The ciliary base acts as a selective diffusion barrier, which is similar in mechanism to the nuclear pore complex that regulates import/export mechanism in the nucleus. Ciliary proteins that are synthesized in the cytosol, are trafficked to the ciliary base for selective import across the diffusion barrier prior to their entry into the cilium. Microtubular transition fibers that separate the cilium from the cell body prevent the free diffusion of cytoplasmic proteins into the ciliary compartment. An additional barrier composed of condensed lipid zone in the periciliary region also separates the cilium. Its likely role is to prevent the free translocation of membrane proteins to the cilium [96]. Recent studies have demonstrated the existence of a septin ring at the base of the cilium. It is composed of Septin 2 (SEPT2) and has been shown to create a diffusion barrier to limit the movement of proteins between the plasma membrane and the ciliary membrane. When SEPT2 was depleted in cells, the proteins of ciliary membrane were found in the plasma membrane surrounding the cilium [32]. However, the physical connection of the septin ring with other structures at the ciliary base is not well understood. Interestingly, SEPT2 is has been reported to be indispensable for cilium-dependent Sonic Hedgehog (Shh) signaling [32].

Although soluble ciliary proteins can reach the ciliary base by diffusion or by moving along the microtubules, membrane proteins have to depend on several transport pathways: (1) They can be directly targeted from the trans-Golgi network (TGN) to the ciliary base, or (2) they can be targeted to recycling endosomes where they undergo sorting followed by transport to the ciliary base, or (3) they can be targeted to the plasma membrane for lateral diffusion into the ciliary membrane. All these ciliary transport pathways are closely integrated with the gating mechanisms that exist at the ciliary base, which allow selective access to only those proteins that are destined to the cilium. Several studies have revealed that targeting of proteins to the cilium requires the presence of specific ciliary targeting motifs or sequences that enable their localization to the cilium [97]. A ciliary targeting sequence was recently identified in Polycystin-2, a protein that localizes to the primary cilium in kidney epithelial cells. Targeting of some proteins to the cilium is phosphorylation-dependent. For example, phosphorylation of Nephrocystin-1 by Casein Kinase-2 is necessary for trafficking to the cilium [98]. There is evidence that IFT proteins may play a role in Golgi to cilium transport. IFT20 has been

shown to localize to the Golgi apparatus where it functions in sorting proteins for the cilium. IFT20-containing vesicles released from the golgi fuse with the base of the cilium to release the ciliary-targeted cargo [99, 100].

1.1.5.5. Regulation of ciliogenesis by the lipogenic factor SREBP1c: Recent research by our team has revealed a novel mode of regulation of ciliogenesis involving lipid metabolism [101]. We have shown that increased lipogenesis, a characteristic feature of cancer cells, was accompanied with the loss of primary cilium. Further research has pinpointed SREBP1c, a key lipogenic transcription factor, as the mediator of these effects. Over-expression of the lipogenic transcription factor SREBP1c in Xenopus and mammalian cell line models resulted in the loss of the primary cilium and subsequent abnormalities in signaling, distrtion of polarized tissue structure and aberrant tissue morphology. One of the downstream SREBP1c targets that mediate cilium loss is a secreted phospholipase [102] that releases fatty acids for the sn-2 position of phospholipids and among others results in increased lysophospholipid levels These findings indicate that altered lipid metabolism as observed in many cancer cells contributes to the, suppression of the primary cilium that often accompanies cancer development (see 1.3) and provide a new mechanism with possible therapeutic potetial.

1.1.6. Timing and cell cycle control of ciliogenesis

The cell cycle and ciliogenesis are interrelated processes. Cell cycle governs the presence of cilia on cells and the timing of ciliary assembly and disassembly is linked to cell cycle progression (figure 1.8). The mother centriole, which forms the basal body for ciliogenesis, is also required for spindle formation during the mitotic phase of the cell cycle. To execute this dual function, the basal body has to be liberated from the cilium before it becomes available for mitotic spindle formation in the cell. The assembly of cilia begins in G1-phase and they are most abundant in G0-phase. In most of the cells, cilia are resorbed as they enter into mitosis [102]. However, resorption of the cilium can also occur prior to S-phase entry or in other parts of the cell cycle phases [102, 103]. Apart from cell cycle stage, the timing of cilium biogenesis is also regulated by centriole age, with the older mother centrioles forming cilia sooner than the daughter centrioles [104]. The molecular switches that govern ciliary assembly and disassembly remain poorly understood. Studies have revealed that centrosomal proteins CP110 and Cep97 are regulators of ciliogenesis. Cep97 recruits CP110 to the centriole, where CP110 caps

the ends of distal centriolar microtubules resulting in the suppression of ciliary assembly. A significant decrease in the levels of both proteins was observed in G0-phase of the cell cycle as compared to levels in a state of proliferative cell division during M-phase. Depletion of either Cep97 or CP110 resulted in abnormal mitotic spindles and formation of cilia-like structures. Conversely, ectopic expression of CP110 leads to the suppression of ciliogenesis even in conditions of serum starvation [105]. Being a target of cyclin-dependent kinases (CDKs), it is plausible that CP110 is phosphorylated in the G1-phase to inhibit its repressive function, thereby allowing ciliogenesis to occur.

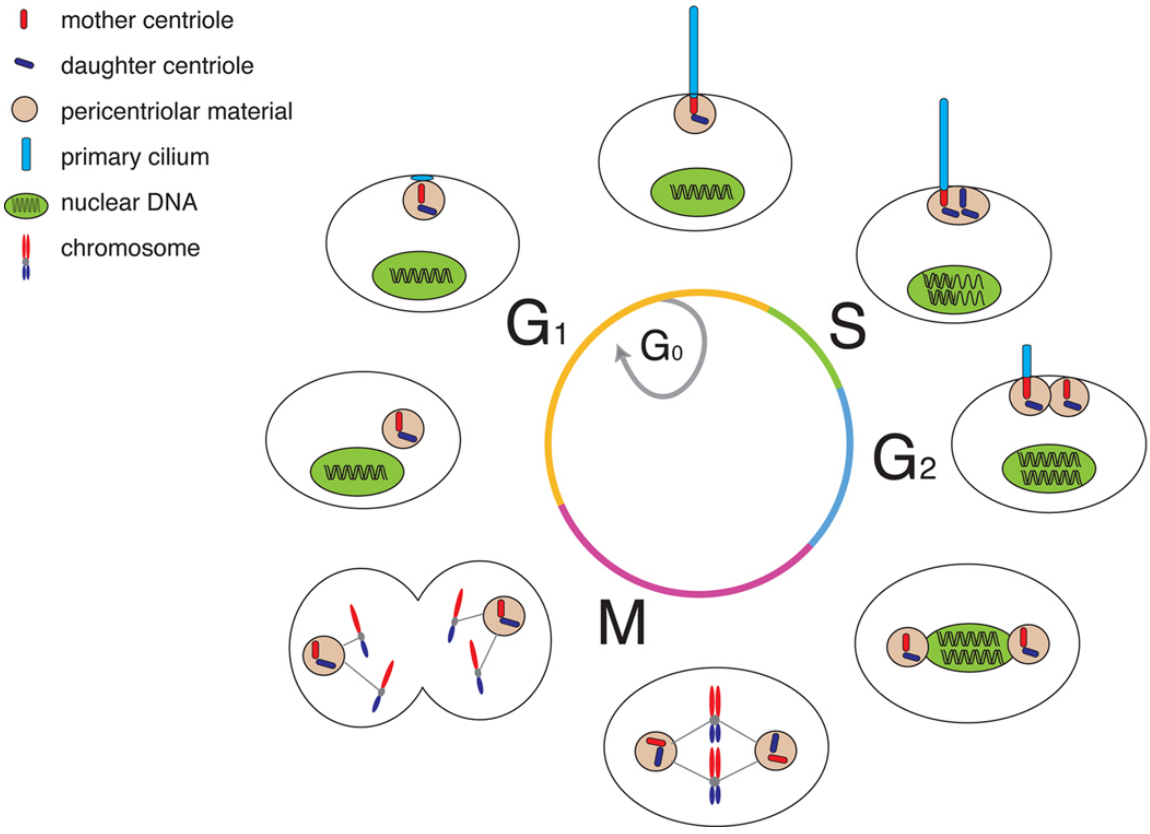

FIGURE 1.8. Dual use of the centrioles during cell cycle and primary cilium formation: In most cells, cilium formation first occurs during G1 following centrosomal docking to the membrane. IFT and accessory proteins build the ciliary axoneme, which extends directly from the mother centriole's triplet microtubules. During this stage of the cell cycle, as well as in G0, the cilium functions as a cellular antenna, interpreting extracellular signals such as Hedgehog and PDGF. Upon entry into S phase, the cell's centrioles and the DNA begin to replicate. The centrioles reach maturity during late G2, at which point the cilium is disassembled so that the engaged centrioles can be liberated for mitotic spindle formation. Once cell division is complete, the centrioles can proceed to ciliary re-assembly in G1. (Figure taken from [241]).

1.1.7. The primary cilium and its significance

Initially dismissed as a vestigial organelle, the primary cilium has now taken the center stage in view of the recent findings that defects in the formation or function of the primary cilium results in a wide variety of human disorders. More than a decade of research has revealed that primary cilia function as sensors of extracellular signals. They capture signals from their immediate surroundings and pass them on to the cell, which is informed of how it should develop itself. Thus, a primary cilium acts as an antenna that serves as a communication device between the cell and the extracellular environment. To facilitate this communication, a large diversity of receptors, channels or proteins can be present on the same cilium. More than a thousand proteins have been found to be either located in the cilium, or associated with the formation or function of the cilium. Mutations or defects in many of these proteins lead to major developmental irregularities in the body, thereby indicating that normal growth and development of an individual is dependent on the structural and functional integrity of the cilium.

1.1.8. The primary cilium as a sensory organelle

The antenna-like geometry and extracellular location of the primary cilium bestow it the unique ability to act as a sensory organelle. Being densely studded with receptors in its membrane, the primary cilium can sense and transmit both physical and biochemical stimuli from the exterior environment to the cell interior. Such stimuli may consist of physical factors like flow and pressure, or chemical substances like growth factors, ligands and morphogens. This results in activation of signaling pathways, which regulate cellular activities such as migration [106], homeostasis [9], division, apoptosis [107], intracellular calcium regulation [108] and cell polarity. Owing to its sensory role, the primary cilium has been designated with a variety of names such as 'cellular GPS' [109], 'cybernetic probe' [110] and 'environmental rheostat [111]. The presence of a large number of specialized proteins enables the cilium to perform diverse sensory roles as discussed below.

1.1.8.1. Fluid flow: Flow sensing ability is one of the most studied functions of the primary cilium. Cilia play an important role in both detection and generation of fluid flow. In the kidneys, bending of primary cilia is essential for the normal functioning of renal tubule cells [112, 113]. The urine passing along the kidney tubules physically bends the primary cilium resulting in an increase in intracellular calcium and thereby

activating a downstream signal. By bending, the cilium senses and interprets fluid shear force as a developmental signal. This mechanosensing function of primary cilia is attributed to a mechanosensor complex consisting of proteins polycystin-1 and -2 that is localized in the cilium [114, 115]. Polycystin-2 has been shown to act as a calcium channel. Activation of Polycystin-2 is dependent on Polycystin-1. Removal of cilia by genetic manipulation or chemical ablation resulted in cells that were mechano-insensitive to fluid flow, supporting mechanosensory function of the cilium [112]. Apart from kidneys, primary cilia are also known to detect fluid flow and serve as mechano-sensors in other organs like liver, heart, pancreas and bone.

1,1.8.2. Left right axis specification: Primary cilia play a vital role in embryonic development by generating fluid movement in restricted embryonic spaces to establish left-right asymmetry [116]. A well-studied example of this phenomenon is in the embryonic node. Two theories have been proposed to explain the role of cilia in the generation of sidedness (left vs right). First is the "morphogen hypothesis", which believes that the node has motile cilia that set up a leftward flow to preferentially transport the morphogens to the left side of the node. The leftward nodal flow is continually refined and reinforced by a circular clockwise rotational motion unique to nodal cilia [117, 118]. The second hypothesis called the "two-cilia model" involves both motile and non-motile cilia at the node [119]. It suggests that motile cilia generate a fluid flow in the leftward direction, which is detected by the non-motile sensory cilia present on the periphery of the node and transduced into an increase in intracellular calcium leading to left-right asymmetry [120]. It is still unclear how exactly this nodal flow is detected and transduced to generate left-right asymmetry. However, recent studies in mouse embryo and fish medaka models argue in favor of the latter hypothesis and raise a tantalizing possibility that both motility and sensory functions of the nodal cilia might dwell in the same organelle [121].

1.1.8.3. Olfactory, photoreceptor and audio sensation: Primary cilia are necessary for sensing odors, light and sound. Cilia present on olfactory sensory neurons can detect and transduce odor through olfactory receptors which are specialized GPCRs present on the ciliary membrane [122]. Activation of these receptors by binding of specific olfactory ligands initiates a signaling cascade through the production of second messenger cAMP within the cilium. cAMP mediates the opening of cyclic nucleotide-gated channel located

in the cilium, leading to the depolarization of olfactory neurons and thereby resulting in the sensation of smell [123, 124]. Olfactory neurons with dysfunctional cilia lack odorant sensation [125]. Recently, it has been discovered that the sensitivity of olfactory cilia is dependent on their length and location in the nasal cavity. A gradient in cilia length exists in the nose where neuronal cells in front of the nose have longer cilia than those in the back. The longer olfactory cilia are more sensitive and are located in the nasal cavity where they can detect more odor-specific molecules [126].

Photoreception is mediated by the primary cilium in the rod and cone cells, which are the actual photoreceptor cells of retina. The primary cilia of these photoreceptor cells have a specialized tip called the outer segment, which contains the photoreceptors that receive light. Upon light stimulation, a signal is initiated by an increase in cGMP, hence resulting in a closure of cGMP channels [127]. This is followed by transduction of visual signals to electrical signals via rhodopsin. The electrical signals are eventually transmitted via the optic nerve to the brain where they are interpreted as an image.

Primary cilia have also been linked to hearing. In drosophila, the cilia of auditory sensory neurons contain two TRP channels that mediate the reception of sound vibrations at the antenna [128]. TRP channels have also been reported to localize to cilia of sensory neurons in *C.elegans*, where they sense and respond to high osmolarity, thereby implicating cilia in osmo-regulation [129].

1.1.8.4. Gravity sensation: Recently, new sensory functions have been attributed to primary cilia. Interestingly, it has been suggested that primary cilia function as transducers of gravitational force [130]. Variability of gene expression levels in rohon beard neurons of zebrafish embryos is linked to cyclic changes in the earth's gravitational field due to the sun and moon. This variability is no longer observed when primary cilia are selectively ablated from the rohon beard neurons. Deciliated neuronal cells subjected to different gravitational forces resulted in homogeneous gene expression. The link between variability in gene expression and changes in earth's gravitational field is restored when neurons regrow their primary cilia.

1.1.8.5. Hunger and satiety sensation: Primary cilia in the central nervous system (CNS) maintain body mass homeostasis by regulating the feeding behaviour and appetite. The cilia of proopiomelanocortin (POMC) neurons present in appetite-regulating part of the brain control hunger and satiation by regulating leptin-mediated pathway

that represses the urge to eat. Ciliary loss or dysfunction in these neurons results in an increase in the feeding behaviour leading to obesity [131].

1.1.8.6. Chemosensation: Chemical signal is another kind of external stimulus that can be sensed by the primary cilium. Localization of specialized receptors to the ciliary membrane renders a chemosensory role to this organelle. It acts as a site to concentrate the receptors. Stationing of important signaling receptors on the ciliary surface instead of general distribution in the plasma membrane increases the likelihood that they will encounter a chemical mediator or agonist. The interaction of a ligand or a chemical mediator with its specific receptor activates the appropriate signaling cascade housed within the cilium. The concentration of receptors on the surface along with the unique housing of these signaling cascades asserts the role of primary cilium as a chemosensor and a critical coordinator of cellular signaling and developmental processes. Examples of proteins and receptors that coordinate cilium-based signal transduction pathways are Patched and Smoothened receptors of Hedgehog signaling [7], Inversin of Wnt signaling [132], PDGFRa [9], Somatostatin receptor 3 [133] etc.

1.1.9. The primary cilium as a hub of cellular signaling

Proper embryonic development and maintenance of tissues and organs relies on the concerted action of diverse signaling pathways that orchestrate intimate interactions between the cells and their environment. It is now a well-established fact that the primary cilium plays a unique role in the coordination of a number of developmental signaling pathways in normal tissues [5]. Signal transduction through primary cilium has two advantages. Firstly, as previously mentioned, it allows the concentration of signal transducers because of the presence of a diffusion barrier between the cilium and the cell body. This enables fine-tuning of contact between the cell and its extracellular environment. Secondly, it allows modulation of signaling pathways by regulating ciliary localization of signal transducers. Depending on the type of signal or pathway involved, specific proteins either translocate to the cilium for their activation, or they can be trapped in the cilium to prevent their active function in the cytoplasm. Thus, by interacting with the extracellular environment and by regulating the entry or exit of signaling proteins, the primary cilium provides a nexus for signaling pathways during tissue growth and development. This section reviews the important signaling pathways as well as some less known ones that have been linked to the primary cilium.

1.1.9.1. Hedgehog signaling: Of all the signal transduction pathways regulated by cilia, the mechanism of sonic hedgehog (Shh) signaling has been best characterized. In mammals, hedgehog signaling plays an important role during embryonic development by regulating a variety of processes that include cell fate specification, regulation of growth patterning, cell proliferation and cell survival [134]. Few examples that illustrate the importance of hedgehog signaling in normal development are in establishment of left-right symmetry [135], patterning of digits in the limbs and skeletogenesis [136], ovarian function and mammary gland development [137], cerebellar development [138], lung and heart and pancreas development [139-141] and formation of neural tube [142]. With the discovery that mutations in cilia affect Shh signaling, a number of experimental studies have been undertaken to firmly establish that presence of cilia is required for proper signaling to occur [6, 143, 144]. Hedgehog is a family of secreted proteins, which include Sonic Hedgehog (Shh), Indian Hedgehog (Ihh) and Desert Hedgehog (Dhh). Binding of these proteins to the transmembrane protein Patch (Ptc) frees the seven-transmembrane receptor Smoothened (Smo) from the inhibitory effect of Ptc, which migrates out of the cilium. The free Smo receptor localizes to the primary cilium [145, 146]. This allows Smo to activate Glioma-associated (Gli) transcription factor to express Hedgehog (Hh) target genes in the nucleus that control responses at different times and in different cell types [147]. Figure 1.9 shows how primary cilia regulate the Hh pathway under normal conditions.

There are three Gli transcription factors. In the absence of Hh ligand, Gli1 is not expressed, Gli2 is inactive and Gli3 is in a repressor form called Gli3R. In the presence of Hh ligand, the pathway is activated and Gli1 is expressed whereas Gli2 and Gli3 are processed by the proteasome. Thus, depending on the absence or presence of Hh, Gli2 and Gli3 proteins undergo post-translational processing into repressor or activator forms respectively. The extent of Hh response is determined by the overall balance of Gli transcription activators and repressors. The primary cilium may serve as a location for the assembly and concentration of protein complexes that are required for proper processing and activation of the Gli transcription factors. [148]. Therefore the cilium regulates the Hedgehog pathway by increasing the local concentration of pathway

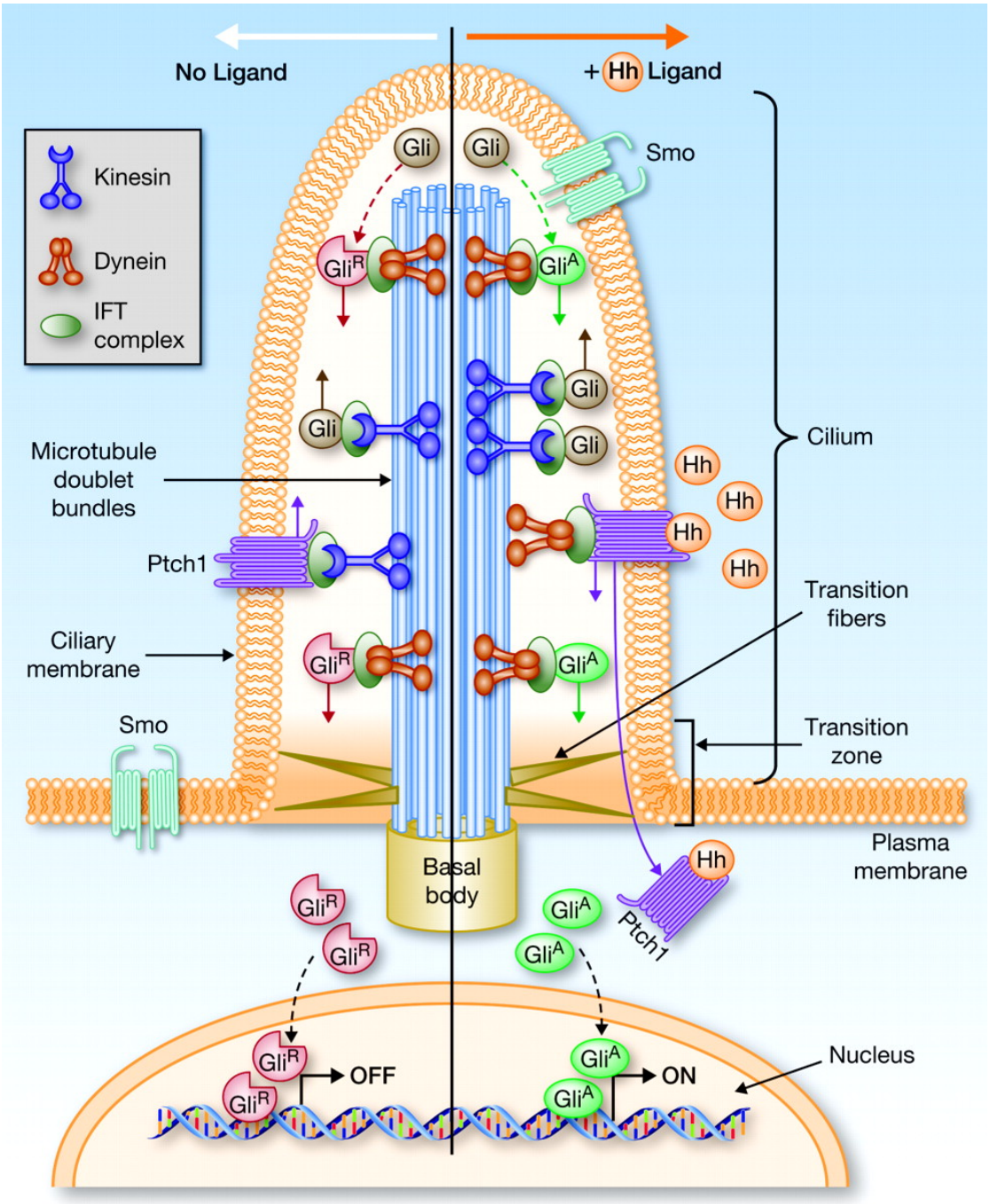

FIGURE 1.9. Regulation of the Hh pathway by primary cilia in normal cells.
Kinesin 2 moves the IFT complex and its cargo (e.g., Gli, Ptch, and Smo) toward the plus end of microtubules (ciliary tip). Dynein 2 moves the IFT complex and its cargo toward the minus end of microtubules (cell body). Hh regulation: In the absence of Hh (left), Gli protein is converted to its repressor form (GliR). Also in the absence of Hh, Ptch1 is localized to the ciliary membrane and Smo is kept out of the cilium. In the presence of Hh (right), Gli protein levels increase in the cilium and Gli is processed into the activator form (GliA) for transport out of the cilium and into the nucleus, where it activates Hh target genes. In the presence of Hh, Ptch1 moves out of the cilium and Smo moves into the cilium, where it promotes formation of the activator form of Gli (GliA). (Figure taken from [281]).

components thereby bringing them together to facilitate key interactions necessary for pathway regulation. It has been shown that Gli proteins and SuFu, a negative regulator of the pathway that binds to Gli proteins, all localize to the distal tip of the primary cilium, suggesting that Hedgehog signaling machinery is located in the primary cilium [148]. Mutations leading to loss of cilia result in abnormal processing of both the repressor and activator forms, confirming the ciliary dependence of this pathway [149]. As Hedgehog pathway is a key regulator of cellular growth and differentiation, abnormal activation of this pathway is a critical factor in the development of a wide variety of cancers. For this reason, manipulation of Hh signaling pathway is being explored as a therapeutic target to potentially delay or cure cancers. Preclinical studies and clinical trials are already underway to test the feasibility of this therapeutic approach in a range of malignancies [150, 151].

1.1.9.2. Wnt signaling: Wnt signaling is a key pathway involved in homeostatic and developmental processes like gastrulation, organogenesis, sex determination, bone and cartilage formation, self-renewal of tissues and hematopoeisis [152]. The vertebrate Wnt signaling is classified into two types: canonical (β-catenin-dependent) pathway and the non-canonical pathway [153]. The canonical Wnt pathway has been implicated in cell differentiation, proliferation, adhesion and survival [154]. Activation of this pathway occurs when the Wnt ligand binds to co-receptors Frizzled (Fz) and LRP, which in turn, activate cytoplasmic Dishevelled (Dvl). Activated Dvl inhibits Glycogen synthase kinase 3 (GSK3) to prevent phosphorylation and destruction of β-catenin [155]. This results in the accumulation of β-catenin in the cytoplasm before it is translocated to the nucleus where it activates T-cell factor (TCF)/lymphocyte enhancer factor (LEF) leading to transcription of Wnt target genes such as *c-Myc, Tcf1 and CyclinD1* [156]. In the absence of Wnt ligand, β-catenin is phosphorylated and destroyed by proteasomal degradation mediated by a destruction complex that consists of β-catenin, Casein Kinase I (CKI), Adenomatous Polyposis Coli (APC), Axin I and GSK3-β [156]. The non-canonical Wnt pathway diversifies into several pathways of which Wnt-calcium pathway and Planar Cell Polarity (PCP) pathway are relatively well studied [153]. The Wnt-calcium pathway has been linked to embryonic dorsal-ventral patterning and in the regulation of cell migration and development [157]. PCP pathway is associated with cell polarity, migration and orientation during embryogenesis and organization of stereo cilia in the inner ear [158, 159]. Non-canonical Wnt signaling is activated when Wnt ligand binds to

Fz receptor, which in turn activates the membrane-bound Dvl, independent of LRP5/6. The activated Dvl regulates RhoA, Rock and Jnk kinases and intracellular calcium levels. Through these downstream effectors, the non-canonical Wnt signaling coordinates Planar Cell Polarity (PCP) and calcium pathways. In the canonical pathway, both cytoplasmic and membrane-bound Dvl can participate whereas only membrane-bound Dvl can activate the non-canonical Wnt pathway [160]. Recent studies have revealed that binding of ciliary protein Inversin (Inv) to cytoplasmic Dvl prevented the activation of canonical Wnt signaling, suggesting that primary cilium may operate as a switch between canonical and non-canonical signaling [8].

1.1.9.3. PDGFR signaling: PDGFR signaling is another pathway acting at the primary cilium. It is important for embryogenesis [161], stimulation of fibroblast migration during wound healing [162], proliferation and apoptosis [163]. Aberrant PDGFR signaling is a causal factor in a broad range of diseases like cancer, fibrosis, vascular disorders and developmental malfunctions [164]. PDGFs and their receptors are synthesized in a wide variety of ciliated cells like epithelial cells, fibroblasts, smooth muscle cells and glial cells [165]. The PDGF pathway comprises of four ligands (PDGF A, B, C and D) and two receptors (PDGFR-α and -β). The receptor PDGFR-α localizes to the primary cilium during growth arrest in mouse embryonic fibroblasts (MEFs) and NIH-3T3 cells [9]. The importance of primary cilia for PDGFR-α signaling was accentuated by demonstrating that this pathway was compromised in ORPK mouse model in which ciliogenesis is disrupted [9]. For the pathway to be stimulated, ligand PDGFA forms a homodimer (PDGFAA) and binds to two PDGFR-α receptors simultaneously, resulting in the activation of Akt and MEK 1/2 pathways in and at the base of the cilium [9]. Further studies show chemotaxis of fibroblasts towards PDGFA ligand, with their primary cilia oriented towards the direction of cell movement during wound healing response. ORPK MEFs with no cilia fail to respond to PDGFA ligand and do not exhibit chemotaxis, emphasizing a significant role of primary cilia in regulation of cell migration [106]. By induction of cell proliferation and maintenance of cells in an undifferentiated state through PDGFR-α signaling, the hESC primary cilium controls pluripotency of embryonic stem cells [166]. In view of its connection to proliferation and apoptosis, PDGFR-α signaling also provides a link between cilia and carcinogenesis.

1.1.9.4. FGF signaling: The primary cilium is also a host to several receptors of fibroblast growth factor (FGF) [167]. FGF signaling is required for normal development as it regulates proliferation, differentiation and migration in a wide array of cells and tissues of both vertebrates and invertebrates. This pathway has been associated with the correct endowment of left-right asymmetry in mice and zebrafish embryos through the creation of morphogen gradients in the embryonic node [168]. Knockdown of fibroblast growth factor receptor FGFR2 and ligands FGF8 and FGF24 in zebrafish tissues resulted in shortening of cilia, implicating FGF signaling in the regulation of cilium length [168]. It has been further shown that FGF signaling controls ciliary length by regulating the expression of genes like ift88, foxj1, rfx2, fibp1 etc. that are responsible for ciliogenesis [168]. Also, dysregulation of FGF pathway or mutations in FGFRs can lead to skeletal defects called craniosynostosis, suggesting a critical role for FGF signaling in the regulation of craniofacial development [169]. Moreover, expression profiling of renal cystic tissues has shown that FGF signaling is upregulated in polycystic kidney disease patients. Further studies are needed to address whether FGF signaling has a general role in cilia formation or its activity is limited to a subset of specialized cilia. Besides, the mechanism by which it regulates the formation or maintenance of cilia is yet to be elucidated.

1.1.9.5. Notch signaling: The notch signaling pathway is known to be involved in the development of many organs, stem cell maintenance and proliferation [170]. The pathway is initiated by binding of notch ligands to the notch receptor, which undergoes two proteolytic cleavage events that promote the downstream activation and transcription of notch target genes [171]. Mutations in the receptor or ligands of notch signaling results in aberrations in tissues and organs like limbs, kidneys, heart, craniofacial region etc. [172]. Recent studies establish that notch signaling is involved in various cilium-associated processes. Notch signaling, which regulates epidermal differentiation during skin development, occurs only in the presence of cilia [173]. Notch3 localizes to the ciliary axoneme in epidermal cells of mouse embryos. Presenilin-2, the catalytic protein that cleaves notch receptor for pathway activation, is found to be concentrated at the basal body. Removal of cilia by knockdown of intraflagellar transport proteins (IFTs) and conditional knockout of genes critical for ciliogenesis, Ift88 and Kif3a during embryogenesis in mice resulted in notch signaling defects and hyperproliferation [173]. Furthermore, over activation of notch signaling results in cilia

elongation, which implicates a role for this pathway in control of cilium length [174]. It has also been shown that notch signaling is involved in senescence [175]. Expression of Notch3 is increased in senescent cells. Moreover, it has been observed that Notch3 expression is decreased in tumor cells and that restoration of this expression resulted in inhibition of cell proliferation and activation of senescence [176]. Collectively, these data provide a link between cilia and notch signaling.

1.1.9.6. Hippo signaling: Recently, Hippo signaling has also been added to the growing list of signaling pathways associated with cilia. This pathway acts as a serine/threonine kinase cascade to regulate cell proliferation and organ size. Nephrocystin-4, a product of nephronophthisis-associated NPHP4 gene has been shown to function as a potent negative regulator of Hippo signaling in mammals [177]. Absence of NPHP4 results in over activation of Hippo pathway leading to a decrease in proliferation. Interaction of Nephrocystin-4 with Lats1 relieves the Lats1-mediated inhibition of TAZ, which consequently moves to the nucleus to mediate cell proliferation through TEAD-mediated transcription [177]. As nephrocystin-4 is present in and at the base of the primary cilium [178], it might act as a connecting link between cell cycle control and ciliogenesis. Some studies hint at a possible role for Hippo signaling in increasing invasiveness and metastatic potential in kidney cancers [179]. Crumb3, a member of the Crumbs receptor family known to affect Hippo signaling, has been shown to localize to the cilium [180]. Further dissection of the underlying mechanism of this pathway is necessary to exploit its therapeutic potential.

1.2. CILIA-RELATED DISORDERS: CILIOPATHIES

Ciliopathies are a heterogeneous group of usually rare diseases resulting from genetic defects that compromise cilia formation or function [181]. These diseases manifest as

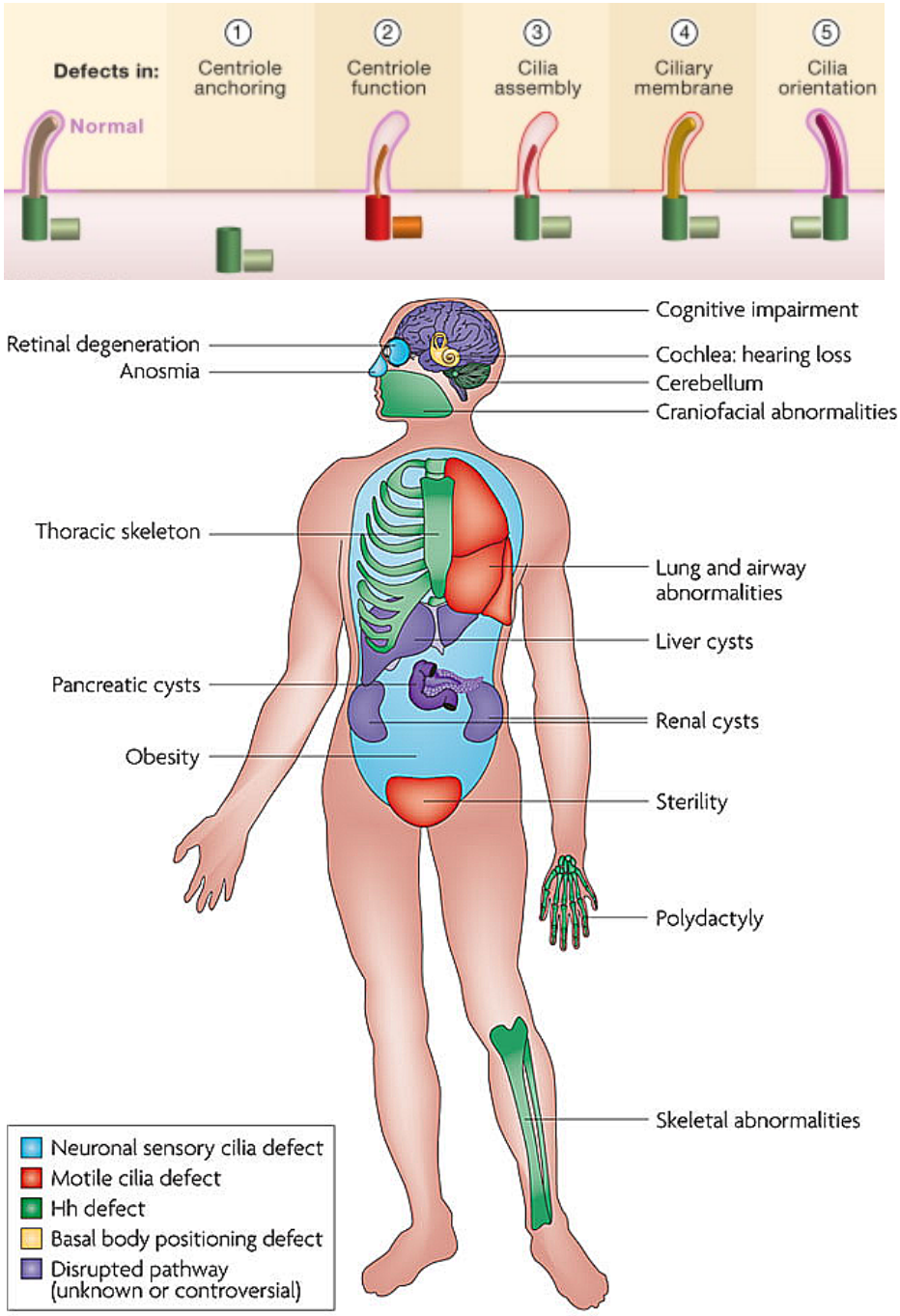

FIGURE 1.10. Cilia defects and ciliopathies: Defects in cilia result in a spectrum of disorders collectively called as ciliopathies. Cilia can be defective due to several reasons. (1) Centrioles are normal, but they fail to migrate, or are not properly anchored, to the cortex. (2) Centrioles may be structurally defective and so unable to initiate proper cilia formation. (3) Cilia assembly may be defective. (4) Proteins normally present in the specialized membrane surrounding the cilium may be defective. (5) Cilia may be positioned in the wrong orientation. The numerous syndromes in humans resulting from either or more of these defects is represented diagrammatically. (Figure taken from [5] & [180]).

either single organ or multisystemic clinical symptoms that include a constellation of features like retinal degeneration, cystic kidneys, mental retardation, polydactyly, skeletal abnormalities, hydrocephalus, hearing loss, obesity etc. [182, 183] (figure 1.10). Given the fact that primary cilia are present on almost all mammalian tissues, ciliopathies often display overlapping clinical symptoms. These symptoms become prominent either at birth or later in childhood, depending on the severity of underlying mutation or on the number of mutations in the ciliary gene. Presently, the spectrum of ciliopathy encompasses more than 50 disorders that include developmental and syndromic diseases. In addition, numerous diseases are being redefined to include cilia as a pathogenesis factor. In principle, following criteria should be fulfilled to consider a disease as ciliopathy [184]

(1) the disease is caused by mutations in genes that encode proteins, that localize to the cilium and have a cilium-specific function;
(2) abnormalities in the structural and functional aspects of the cilium have been demonstrated in patient-derived tissue or an animal model of the disease; and
(3) the disease is characterized by abnormalities of the types associated with a known ciliopathy.

If a disease satisfies either one or two, but not all the three criteria, it is classified as a suspected but not proven ciliopathy. The rapidly expanding list of suspected ciliopathies highlights the importance of understanding the role of cilia in human health and disease. The predominant organs that are affected by ciliary dysfunction include the kidney, eye, brain and liver. Ciliopathies can arise from defects in either motile or sensory functions of cilia. A number of well-known and some lesser-known ciliopathies are discussed below.

1.2.1. Primary Ciliary Dyskinesia: The first ciliopathy to be identified was primary ciliary dyskinesia (PCD), also known as immotile cilia syndrome, which is caused by defects in motile cilia [185, 186]. Patients with PCD are characterized by chronic bronchitis and sinusitis due to the failure of cilia to clear mucus and inhaled bacteria out of the respiratory tract. This defect in mucociliary clearance leads to recurrent infections by pathogenic bacteria like *Haemophilus influenzae*, *Staphylococcus aureus, Streptococcus pneumoniae* resulting in recurrent pneumonia, bronchiectasis and irreversible dilation

of bronchi, which severely impairs respiratory function. Male infertility and reduced fertility in women are commonly observed as this disorder also affects the motility of sperm flagella and cilia of fallopian tubes. Moreover, the patients develop hydrocephalus, which is enlargement of the head due to abnormal accumulation of cerebrospinal fluid resulting from defective motility of ependymal cilia present in the brain [187]. Almost half of the PCD patients display situs inversus, also known as Kartagener's syndrome [188]. It is a developmental defect in which the positions of the heart and other internal organs are reversed forming a mirror image of the normal positioning. This happens due to the impaired motility of nodal cilia that initiate the normal left-right asymmetry in early embryo [189]. Currently, more than 28 PCD genes have been identified [190]. Almost 50% of the PCD patients have defects in genes that encode proteins of the outer dynein arm that is necessary for ciliary motility. Hence, these genes make an attractive target for genetic screening of PCD. In general, mutations that affect motile cilia lead to a set of symptoms that are different as compared to those affecting sensory cilia (primary cilia).

1.2.2. Polycystic Kidney Disease: In humans, polycystic kidney disease (PKD) is the most common life-threatening ciliopathy. It manifests in two major forms namely autosomal dominant and autosomal recessive. The most prevalent form of PKD is the autosomal dominant polycystic kidney disease (ADPKD), which usually affects adults and has an incidence of 1:1000. The disorder is characterized by grossly enlarged kidneys, predisposition to renal stones and development of renal cysts leading to kidney failure. The end-stage patients may require therapy by dialysis or kidney transplantation. Apart from kidneys, cysts are also formed in the liver, pancreas and intestine of ADPKD patients along with cardiovascular defects, hypertension and recurrent urinary tract infections. Autosomal recessive polycystic kidney disease (ARPKD) is more common in neonates and children. It has an occurrence of up to 1 in 6000 live births and has a high mortality rate in affected newborns. Characteristic features include renal cystic degeneration, dilation of collecting ducts of kidneys and liver fibrosis. The causative genes of ADPKD are PKD1 and PKD2 that encode polycystin-1 (PC-1) and polycystin-2 (PC-2) respectively [191]. Mutation in either of the two genes can lead to ADPKD although mutation in PKD1 is found in more than 85% of all cases. ARPKD is caused by mutation in a single gene PKHD1 that encodes the protein fibrocystin. Interestingly, PC-1, PC-2 and fibrocystin have been shown to localize to the

primary cilium [36, 192]. PC-1 and PC-2 interact with each other to form a calcium permeable channel on the ciliary surface that is required to mediate a flow-dependent calcium response [114]. Owing to the presence of these calcium channels on their surface, primary cilia act as flow sensors in the lumen of renal tubules. Deflection of cilia in response to renal fluid flow triggers the entry of calcium into the cells, which is necessary for the activation of signaling pathways that control kidney epithelial cell differentiation and proliferation [193]. Therefore, mutations in these proteins or failure in the assembly of cilium as a whole results in the disruption of ciliary signaling leading to PKD.

1.2.3. Bardet-Biedl Syndrome: Bardet-Biedl syndrome (BBS) is a multisystemic disorder with primary features that include obesity, renal anomalies, hypogonadism, mental retardation, developmental delay, polydactyly and retinal degeneration [194]. Almost all patients with BBS are obese, which may be linked to dysfunctional cilia of neurons present in the satiety center of hypothalamus [195]. BBS is a ciliopathy with highly variable clinical features. Secondary features include anosmia, diabetes, ataxia, asthma, cardiovascular anomalies and occasionally situs inversus. The leading cause of mortality in BBS patients is kidney failure due to cystic tubular disease and anatomical malformations. BBS is characterized by genetic heterogeneity as it is caused by mutations in one of the 18 genes (BBS1 to BBS18) that are highly conserved in ciliated organisms. Symptoms can differ depending on which of the causative genes is mutated. However, genotype-phenotype correlations have not been consistently established yet. For example, human BBS patients often exhibit polydactyly but it has not yet been reported in mutant mice. All the BBS genes encode primary cilia proteins that localize to the centrosome, basal body or ciliary axoneme and are involved in the maintenance of axonemal microtubules and in the coordination of cell cycle. Eight of the BBS proteins combine to form an octameric complex called BBSome, which has been implicated in IFT assembly and in the localization of certain receptors to the primary cilia in neurons [196, 197]. The localization of BBSome to the centriole at the base of cilium depends on the interaction of BBSome with proteins expressed at centriolar satellites [198]. The interaction of the BBS4 component of BBSome with centriolar satellite protein CEP290 is necessary for the recruitment of BBSome to the cilium. Mutations in CEP290 have been shown to disrupt BBSome formation and its localization to the cilium [198, 199]. Recent studies have identified that the interaction between BBSome and CEP290 is

mediated by another centriolar satellite protein SSX2IP, which is needed to target BBS2, BBS4, BBS8, CEP290, SSTR3, RAB8A to the cilium [200]. Other studies show that Nephrocystin-5 (NPHP5) protein and CEP290 interact with each other in the transition zone and both can bind BBSome proteins to alter their localization. However, their interaction with the BBSome appears to have different effects on BBSome stability [201]. It has also been shown that BBSome binds with ARL6 to form a coat on the surface of vesicular membrane lipids in the cell [85]. A recent study has revealed that this coat allows the sorting of membrane proteins into the cilium through recognition of ciliary targeting sequences by the BBSome [202].

1.2.4. Orofaciodigital Syndrome: Orofaciodigital Syndrome type 1 (OFDS1) is an X-linked dominant disorder caused by mutations in the gene OFD1, which encodes a centrosomal protein that localizes to the basal body and is essential for the organization of the centrosome and regulation of the cilium [203]. It is a male lethal disorder in which affected males die in utero. The disease is characterized by malformations of the oral cavity, facial dysmorphologies, digit abnormalities, cystic kidneys and abnormalities of the central nervous system (CNS) with accompanying mental retardation [204]. OFDS1 shares phenotypic similarities with other forms of this syndrome (OFD type II to XIV). In recent years, 11 different causative genes of OFDS have been identified, most of which have been proven to be involved in primary cilia or basal body function [205]. The genes TMEM231 (OFDIII), TMEM216 and TMEM107 (OFDIV) share an overlap with Joubert and Meckel Gruber Syndromes. C2CD3, the causative gene of the recently identified OFD type XIV has been shown to be responsible for centriole elongation, suggesting that regulation of centriole length is likely to gain focus as an emerging mechanism in ciliopathies [206]. Another recent study shows that KIAA0753/OFIP protein, which is conserved in ciliated species, was found to be mutated in a patient with OFDVI syndrome [207]. The authors further showed that the mutated KIAA0753/OFIP loses its capacity to interact with FOR20 and OFD1 causing defective recruitment of components to centrosomes and satellites, which might be the molecular basis of the defect causing OFDVI syndrome.

1.2.5. Joubert Syndrome: Joubert syndrome (JBTS) is a group of disorders characterized by mental retardation and ataxia due to hypoplasia of the cerebellar vermis, hypotonia, severe psychomotor delay, episodes of rapid breathing, and

oculomotor apraxia. The other common features include cystic kidney disease, nephronophthisis, hepatic fibrosis, polydactyly, retinal degeneration, endocrine abnormalities and craniofacial dysmorphologies. JBTS is diagnosed by the appearance of characteristic "molar tooth sign" on magnetic resonance imaging, a consequence of underdevelopment of cerebellar vermis and abnormal formation of midbrain and hindbrain [208]. The syndrome is associated with multiple causative genes resulting in genetic heterogeneity that brings variability and overlap of features in JBTS and other ciliopathies. Based on the underlying gene responsible for the disease, JBTS is classified into 21 types (JBTS type 1 to 20 and one unassigned yet). Causative genes that are mutated include, INPP5E (JBTS1), TMEM216 (JBTS2), AHI1 (JBTS3), NPHP1 (JBTS4), CEP290 (JBTS5), TMEM67 (JBTS6), RPGRIP1L (JBTS7), ARL13B (JBTS8), CC2D2A (JBTS9), OFD1 (JBTS10), TTC21B (JBTS11), KIF7 (JBTS12), TCTN1 (JBTS13), TMEM237 (JBTS14), CEP41 (JBTS15), TMEM138 (JBTS16), C5Orf42 (JBTS17), TCTN3 (JBTS18), ZNF423 (JBTS19), TMEM231 (JBTS 20), TCTN2 (JBTS-unassigned), most of which are known to localize to the primary cilium or basal body. Emerging studies show that many more genes involved in JBTS are being identified at a fast pace. Different groups of researchers have identified mutations in genes CEP104, CEP120, CSPP1, KIAA0556 and KIAA0586 that lead to Joubert syndrome [209-213]. The transition zone protein TMEM107 has recently been reported to cause Joubert Syndrome by recruiting other ciliopathy proteins to the transition zone [214]. Almost 50% of the JBTS patients have mutations in CEP290, which may be attributed to the large number of proteins that CEP290 interacts with [215]. Involvement of ARL13B points towards a possible role for primary cilia-mediated hedgehog signaling in the pathogenesis of JBTS [216]. The disease shares a genetic overlap of at least 13 causative genes with another ciliopathy Meckel syndrome (MKS).

1.2.6. Meckel Syndrome: Meckel Syndrome is a lethal autosomal recessive multi-organ ciliopathy characterized by perinatal lethality, fibrotic changes in the liver, renal cysts, occipital encephalocele, cardiac abnormalities, central nervous system defects, polydactyly and cleft palate. Patients with Meckel Syndrome (MKS) invariably die of renal or respiratory failure. So far, MKS has been classified into 13 types based on the following causative gene for each type: MKS1 (MKS1), TMEM216 (MKS2), TMEM67 (MKS3), CEP290 (MKS4), RPGRIP1L (MKS5), CC2D2A (MKS6), NPHP3 (MKS7), TCTN2 (MKS8), B9D1 (MKS9), B9D2 (MKS10), TMEM231 (MKS11), KIF14 (MKS12) and

TMEM107 (MKS13). All of these genes are associated with ciliary functions. MKS proteins are involved in functions like establishment of barrier at the base of cilium during early ciliogenesis, basal body docking, maintenance of normal Shh signaling in neural tube and the developing limb [26, 37, 42]. Most of the morphological defects associated with MKS type1 have been attributed to aberrant Hedgehog signaling [217]. MKS3, also known as Meckelin, is structurally similar to Frizzled receptor and could possibly play a direct role in non-canonical Wnt signal transduction although it is yet to be proven [218].

1.2.7. Monosomy 1p36: Monosomy 1p36 is a terminal deletion syndrome caused by deletion of the distal tip of the short arm (p) of chromosome 1, which is rich in many genes that include Rer1p. Recent evidence shows that Rer1p, an ER-Golgi cargo receptor protein is involved in the regulation of ciliary length and function. Loss of Rer1p results in shortened cilia and defective sensory function of cilia, leading to a number of clinical characteristics similar to those associated with monosomy 1p36 [90]. Hence, monosomy 1p36 appears to be another addition to the ever-growing list of ciliopathies. The frequency of occurrence of this syndrome is estimated to be 1 in every 5000 births. The syndrome is characterized by distinctive facial dysmorphic features, intellectual disability, developmental delay, hypotonia, seizures, hearing impairment and heart defects. The severity of the syndrome varies between the patients. Although some patients reach adulthood, data on projected lifespan is lacking as it is a recently discovered syndrome.

1.2.8. Birt-Hogg-Dube Syndrome: Birt-Hogg-Dube (BHD) Syndrome is an autosomal dominant disorder caused by inactivating mutations in the FLCN gene that encodes for a tumor suppressor protein called folliculin [219]. Lung and kidney cysts, benign skin tumors and predisposition to kidney cancer characterize this condition. It is thought that FLCN might act as a tumor suppressor in the kidney as a majority of renal tumors show a somatic loss of the wild-type allele [220]. Recent studies have revealed that FLCN interacts with binding proteins FNIP1 and FNIP2, which cooperate with FLCN to suppress kidney tumors [221]. The authors show that double inactivation of Fnip1 and Fnip2 leads to enlargement of polycystic kidneys or the development of kidney cancer, which mimics the Flcn-deficient kidney phenotypes. FLCN has also been implicated in the nucleocytoplasmic shuttling of TDP-43, a protein whose nuclear loss in combination

with cytosolic accumulation initiates the early stages of neurodegeneration [222]. Since BHD exhibits characteristics of ciliopathies, it has been speculated that FLCN might have a ciliary role. Studies have proved that FLCN localizes to cilia, centrosomes and mitotic spindle [219]. Alterations in the levels of FLCN result in changes in the onset of ciliogenesis rather than abrogation of cilia. The observed symptoms of BHD are partly attributed to the deregulation of canonical Wnt signaling due to abnormal ciliogenesis. BHD provides an interesting link between ciliopathies and cancer, providing strength to the argument that certain cancers might be a part of the ciliopathy spectrum.

1.3. THE PRIMARY CILIUM AND CANCER

It has been recently contemplated that defects in the biogenesis of primary cilium might be a crucial step in the development of cancer. This hypothesis stems from the fact that cilia not only have the ability to influence cellular signaling pathways but also to physically alter the progression of cell cycle. Plenty of evidence exists to show a compelling link between primary cilia dynamics and the development of cancer. Several reports document the loss of primary cilia in cancer cell populations of different types of tissues. Because the primary cilium regulates signaling pathways, the loss of this organelle may contribute to aberrant signaling leading to cancer progression. The different aspects of the primary cilium in development of cancer are discussed below in more detail.

1.3.1. Role of the primary cilium in cancer predisposition syndromes

Emerging studies on the role of cilia in familial hereditary syndromes have revealed a predisposition of these syndromes to cancer development. Interestingly, several well-established tumor suppressor proteins have been identified in these syndromes, which are involved in the biogenesis of cilia in addition to their attributed functions. This association between tumor suppressor proteins and cilia suggests that ciliary loss could be an early event in tumorigenesis. For example, the familial cancer syndrome von Hippel-Lindau disease (VHL) is caused by mutations in the VHL tumor suppressor, which is required for the formation of primary cilium in conjunction with GSK-3β. This syndrome is characterized by renal and pancreatic cysts, clear cell renal cell carcinoma (ccRCC), retinal angiomas, tumor in the adrenal gland, cysts and hemangiomas in the central nervous system [223]. Similarly, mutation in APC tumor suppressor gene causes familial adenomatous polyposis (FAP), a spectrum of diseases that includes Gardner's

syndrome which shows manifestations like desmoid tumors, osteomas and skin cysts [224]. APC protein binds to kinesin-2 and stabilizes microtubules [225]. Another example is Gorlin syndrome, which is caused by mutations in the hedgehog components like Ptch1 or SuFu that are associated with cilia [226]. Characteristics of this disorder include predisposition for basal cell carcinoma (BCC) and medulloblastoma. Tuberous Sclerosis Complex (TSC) is an inherited genetic syndrome associated with the formation of benign tumors in the brain, kidneys, lungs, eyes, heart and skin [227]. Normally, TSC-associated tumors are not malignant but those that are malignant usually affect the kidneys. The disease is caused by mutations in either TSC1 or TSC2 genes that code for tumor suppressors harmarin and tuberin respectively [228]. Loss of TSC1 or TSC2 enhances cilia length in fibroblasts and renal tubules [229]. A recent study has demonstrated that interplay between PKD and TSC genes leads to renal cyst formation in a TSC mouse model through negative regulation of polycystin-1 (PC-1) by mTORC1, which also impairs the trafficking of PC-1/2 complex to the cilium [230]. Therefore, the predisposition of TSC to renal cancers is possibly linked to PKD. These results also open up a new perspective for the use of mTOR inhibitors like rapamycin for the treatment of PKD and TSC. Whole-exome sequencing studies have revealed that predisposition to nasopharyngeal carcinoma might be linked to a cilia-related gene MST1R, which is important for ciliary motility in the epithelial cells of normal nasopharyngeal mucosa [231].

To explain the role of cilia in the development of cancer in familial syndromes, it is generally agreed that a mutation occurs or is inherited which directly affects cilia or a pathway associated with ciliary function. The loss of normal ciliary function leads to increase in proliferation, which may result in immediate tumor formation depending on the function of mutated gene involved or on the sensitivity of the cell to ciliary signaling. In other cases, increased proliferation results in pre-cancerous benign growths that are unstable and predispose the cells to further mutations that ultimately lead to tumor formation.

1.3.2. Is cancer a ciliopathy?

The development of cancer does not depend on one single event, but rather is a multi-step process that requires the cell to acquire several capabilities that allow survival and growth. The cancer cell achieves these required capabilities by promoting chromosomal

instability, which leads to the accumulation of multiple germline and somatic mutations over a course of several years [232]. Thus, mutations leading to the loss of cilium alone cannot be a sufficient requirement to drive or prohibit tumorigenesis. Although many ciliopathies involve cell cycle defects, not all ciliopathies predispose to cancer. Therefore, it would be incorrect to proclaim that cancer is a ciliopathy although it overlaps with many disorders of the ciliopathy spectrum.

1.3.3. Clinical correlation between primary cilia and cancer

A correlation between the loss of primary cilium and cellular transformation has been observed for more than a decade [233]. Based on clinical evidence, it is widely accepted that loss of primary cilia is a common feature of cancers. A number of reports show that many types of cancers tend to lose the cilium. This supports the line of scientific thinking that cilia might act as tumor suppressors. However, recent reports provide evidence that some types of cancers appear to be dependent of the presence of cilium, suggesting a tumor-promoting role of cilia. Since primary cilia are present in most tissues of the human body, ciliary frequencies have been evaluated in several tissues and their corresponding tumor types.

1.3.3.1. Tumors dependent on ciliary loss

(i) Breast cancer: Microscopic analysis of breast cancer tissues and cell lines derived from breast cancer revealed decreased ciliary frequencies as compared to normal breast tissue epithelia and fibroblasts [234]. Cell lines derived from aggressive cancer tissue showed severely reduced cilia frequencies even upon prolonged serum starvation. However, this was shown to be independent of increased proliferation as determined by Ki67 staining [234, 235]. Several cilia-associated genes like Gli1, RPGRIP1 and DNAH9 are known to be mutated in breast cancers [236]. Moreover, the gene NPHP9 (NEK8), which is a known modulator of ciliary length, is upregulated in breast cancers [237].

(ii) Kidney cancer: In these cancers, ciliary dysfunction usually leads to the development of renal cysts, which is a hallmark characteristic of many classic ciliopathies. The formation of renal cysts is caused in part by the deregulation of canonical Wnt signaling and planar cell polarity (PCP) pathways [238]. Mounting evidence suggests that such cystic lesions increase the risk of renal cancer [239]. Interestingly, Von Hippel Lindau (VHL) patients display complex renal cysts that can be considered as premalignant tumors. VHL, a tumor suppressor gene has been well

characterized for its role in the maintenance of cilia. Analysis of a cohort of kidney tissue samples from renal cell carcinoma patients revealed a marked decrease in the ciliary frequency in tumor tissue as compared to adjacent normal parenchyma tissue [238]. This observation suggests that primary cilia are predominantly lost in renal tumorigenesis and this ciliary loss might possibly participate in malignant tumor development.

(iii) Pancreatic cancer: Loss of cilia has been reported in human pancreatic ductal adenocarcinoma (PDAC) [240]. Reduced ciliary frequencies were observed in both pancreatic cancer cells and pancreatic intraepithelial neoplasia (PanIN) lesions in human patients. Loss of cilia was observed to occur in the early stages of tumor development [241]. Primary cilia were absent even when the cells were not actively proliferating. PDACs are also characterized by activated kras signaling [242]. Ciliogenesis was restored in a mouse pancreatic cancer cell line upon inhibition of oncogenic kras pathway, thereby indicating a role of kras in cilia repression [240].

(iv) Colorectal cancer: The role of primary cilia is beginning to emerge with reports of mutations in several cilia-associated genes in this type of cancer. Aurora A, a kinase that promotes ciliary disassembly is commonly mutated in colorectal cancers [243]. Intriguingly, the gene PKHD1, which is often mutated in polycystic kidney disease (PKD) has also been found to be mutated in colorectal cancers [244]. Furthermore, mutations in Gli3, a member of the Hedgehog pathway, have also been observed in these cancers [245]. Recent studies have shown that reduction in the number of primary cilia in colon tissue is strongly associated with an increase in the proliferation of colon epithelial cells [246]. Put together, these pieces of evidence converge to a role of primary cilia in the pathogenesis of colorectal cancers.

(v) Ovarian cancer: Aberrant Hedgehog and PDGF signaling has been reported in ovarian cancer cells originating from the ovarian surface epithelium [247]. Perturbation of these two critical signaling pathways is associated with defects in the primary cilia of these cells. Suppression of ciliogenesis was observed even under proliferation-limiting conditions created by growth arrest of cells [247]. Ovarian cancer cells also show higher levels of Aurora A as compared to corresponding normal cells. Given that these cancers have low cilia frequencies, it can be deduced that higher levels of Aurora A are involved in the suppression of ciliogenesis. Co-evolutionary analysis of ovarian serous

cystoadenoma has revealed an overrepresentation of mutated cilia genes in this tissue [248]. Based on this observation, the authors postulate that deregulation of the ciliary network of proteins will result in proliferation and cancer development [248].

(vi) Melanoma: In melanomas, the presence of cilia was evaluated at different stages of development. Whereas primary cilia were present on normal melanocytes, very few cilia were present in early melanomas in situ [249]. Cilia are completely lost as the tumor progresses to invasive and metastatic phases. This study demonstrates that primary cilia are progressively depleted in cutaneous melanoma and establishes the loss of primary cilia as an early phase of melanoma development [249]. A similar trend was observed in dysplastic melanocytic nevi where the loss of cilia correlates with increasing degree of dysplasia [250]. Recent studies show that the loss of cilia in melanomas is independent of increased proliferation and cell cycle progression [251].

(vii) Prostate cancer: Primary cilia are lost through prostate cancer progression. A decrease in the percentage of ciliated cells was observed in both preinvasive and invasive prostate cancer tissue as compared to normal tissue [252]. The few visible cilia on the cancer tissue appeared shorter and dysfunctional, as assessed by measurement of cilia length, an indirect measure of functionality. Hh and canonical Wnt pathways have been reported to be upregulated in prostate cancers [252, 253]. Loss of cilia has been attributed to the increased activation of these pathways, which result in the progression of prostate cancer.

1.3.3.2. Cilium-dependent tumors

Some studies have provided evidence that cilia are present in certain cancer-types giving rise to the notion that they may play a role in the promotion of such cancers. Primary cilia have been detected in glioblastoma tumor biopsies and primary cell lines in a subpopulation of cells that were more proliferative, invasive and resistant to therapeutics [254]. Basal cell carcinomas (BCC) and medulloblastomas have been shown to be heavily dependent on cilia for their growth [255, 256]. It has been demonstrated that KIF3A-mediated knockdown of cilia inhibits the growth of medulloblastoma and basal cell carcinoma cells [257]. Primary cilia were also reported to be present in around 25% of pancreatic ductal adenocarcinoma tumor samples and the presence of cilia correlated with poor prognosis [258]. There appears to be a range in the percentage of tumor samples with cilia and a range in the percentage of cells with cilia in an individual

tumor. These ranges should be further explored to correlate specific levels of cilia frequency with specific cancer subtypes and response to treatment. Future studies should focus on the separation of cancer cell populations with and without cilia to compare the differences between them.

1.3.4. The primary cilium as a tumor promoter

Recent studies have shown that via regulation of Hh pathway, cilia can either promote or prevent tumorigenesis. The outcome is dependent on the nature of the initiating event i.e. where in the Hh pathway the oncogenic mutations occur [256, 257] (figure 1.11). If activating mutations occur downstream of the Hh pathway (affecting Gli2), presence of primary cilia is not required for Hh activation and tumor development. On

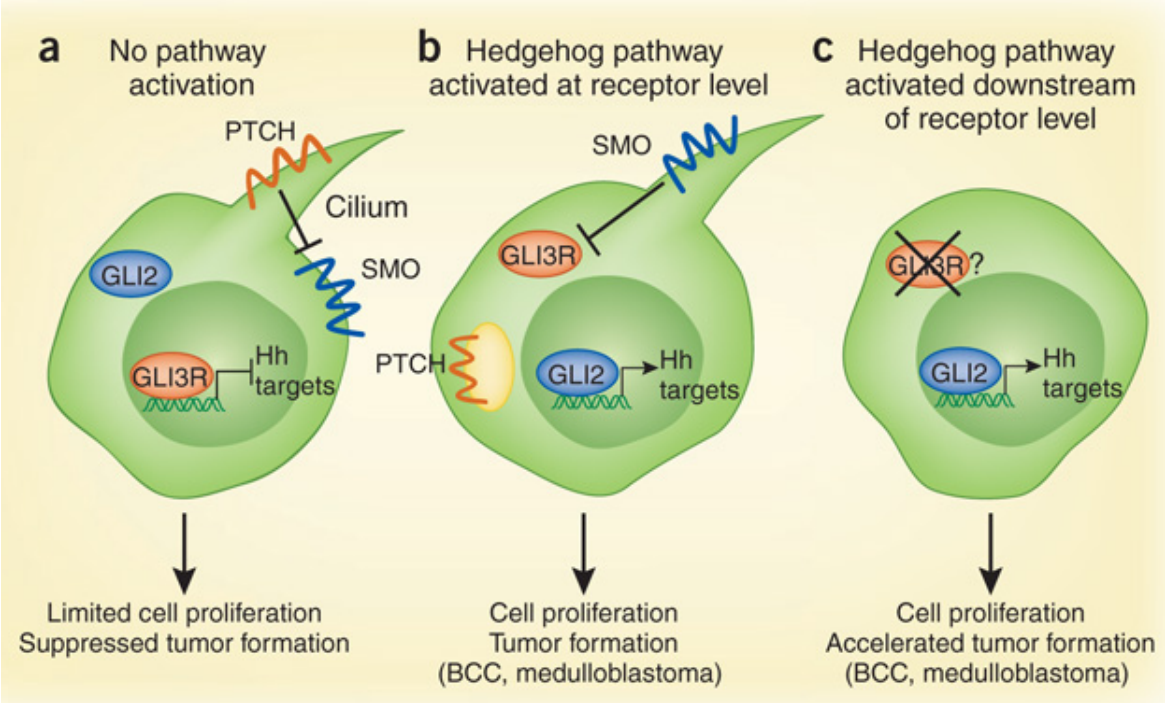

FIGURE 1.11. Dual role of primary cilia in Hedgehog (Hh)-induced tumor development: (**a**) In the absence of ligand, the PTCH receptor is localized to the cilia, whereas the SMO co-receptor is excluded from the cilia. The GLI3 repressor (GLI3R) prevents activation of target genes. (**b**) In the presence of primary cilia, the mutant oncogenic Hedgehog co-receptor SMO constitutively localizes to this organelle, inhibits formation of GLI3R and activates the downstream GLI transcription factors (for example, GLI2). GLI2 stimulates target gene activation, promoting increased cell proliferation and tumor development. (**c**) Activation of GLI2 can also preferentially stimulate tumor development in the absence of cilia. The authors propose that GLI3R is downregulated in these cells, but the mechanism is still unclear. (Figure taken from [253]).

the contrary, when mutations occur at the receptor level (Smoothened or Patched), primary cilia are needed for Hh signaling and consequent tumorigenesis. Epithelial cancers like ovarian, colon and pancreatic cancers harbor mutations downstream of Hh

pathway resulting in indirect activation of Gli2. Loss of cilia in such cancer types accelerates the growth of tumors indicating that primary cilia function as tumor suppressors in Gli2-expressing cells [256, 259]. In contrast, subtypes of cancers that are heavily dependent on hedgehog signaling, like basal cell carcinoma, medulloblastoma and glioblastoma are induced by an activated form of smo and are thus dependent on the presence of cilia for their proliferation and survival [256, 257, 260]. Removal of primary cilium strongly inhibits the growth of these tumors. Notably, the role of primary cilia may extend beyond Hh-driven tumorigenesis, which is evidenced by the fact that primary cilia are also present on human medulloblastomas that are driven by Wnt activation [256, 261, 262]. Remarkably, some human medulloblastomas display primary cilia whereas others do not. In fact, primary cilia are exclusively present in medulloblastomas that arise due to activations in Hh or Wnt signaling but not in medulloblastomas that belong to other distinct molecular subgroups.

1.3.5. The primary cilium as a potential tumor suppressor

Given the ability of primary cilia to influence cell cycle and to regulate cilia-related signal transduction, any defect, dysfunction or loss of cilia has been considered to be an indispensable step for cancer development [263]. It has been proposed that the loss of cilia in cancer cells reduces or alters the responsiveness of cells to extracellular cues that regulate cellular growth and differentiation [111]. Cancer cells lacking cilia are thus insensitive to environmental repressive signals. Many recent studies have shown that a large number of receptors for critical signaling pathways and adhesion molecules are clustered on the surface of the cilium, which necessitates the presence of an intact cilium for normal function. These include components of several pathways like Sonic Hedgehog (Shh), platelet derived growth factor receptor a (PDGFRα), planar cell polarity (PCP), von Hippel-Lindau and Glycogen Synthase Kinase 3β (VHL/GSK3B) pathways [9, 146, 264]. Therefore, primary cilia are necessary from both structural and signaling perspective for the normal growth and development of a cell.

Although defective cilia have always been associated with cancer, a direct role of cilia in tumorigenesis is yet to be established. The first molecular link between ciliary disassembly and cell cycle progression was provided by Snell and co-workers who demonstrated that the disassembly of cilia in chlamydomonas was mediated by the protein CALK, an Aurora kinase that promotes cell cycle [265]. Later, the group of

Golemis replicated this finding in mammalian cells. They found that shortening of cilia in mammalian cells is also mediated through Aurora kinase-dependent pathway [17]. In the presence of serum, HEF1 activates Aurora A, which phosphorylates HDAC6 to promote disassembly of cilia followed by cell cycle re-entry [17, 266]. Moreover, loss of genes that encode proteins required for ciliary assembly leads to cancer-promoting conditions like defects in planar cell polarity, constitutive activation of Wnt, TGF-β, MAPK signaling, deregulation of Hh signaling, EGFR and c-Myc accumulation, cystic disease, epithelial hyperplasia and metaplasia [60, 267-271]. Increased lipogenesis is a feature observed in many cancers [272]. The lipogenic transcription factor SREBP1c is often aberrantly activated in many cancers. Our group has recently shown that loss of cilia is mediated by the lipogenic transcription factor SREBP1c (see also 1.1.5.5.) [101]. Based on these observations, it would be reasonable to expect that dysfunction or loss of the cilium may trigger the development of tumors and malignancies. However, it cannot be ruled out that cilium resorption may occur as a physiologic consequence of cell cycle progression and the loss of cilium might merely represent the enhanced rate of cell division. Although transformed cells usually lack cilia, no clear picture has emerged yet to ascertain whether this lack is a cause or consequence of transformation. It still remains a long-standing unresolved question.

Ciliogenesis and cell division are considered as events that are mutually exclusive. Because the centrioles must be released from the basal body to function as a mitotic spindle, a causal relationship exists between cilia and cell cycle [33, 103]. Based on studies on trichoplein [76] and Nek2-Kif24 [273], a model was proposed which requires a continuous suppression of primary cilia formation in proliferating cells for proper cell cycle progression to occur [274]. Thus, from a structural perspective, the physical presence of cilium on a cell can act as a brake to prevent cell cycle re-entry [275]. This concept in which the cilium functions as a physical check point to hamper the progression of cell cycle is substantially supported by evidence from studies on Nde1 and Tctex-1 [69, 81]. Knockdown of Nde1, a centrosomal protein, results in elongated cilia in mammalian cells. Interestingly, cells with longer cilia due to Nde1 depletion exhibited a delay in cell cycle re-entry after serum addition. Co-depletion of Nde1 with either Ift88 or Ift20, two essential genes for ciliogenesis, reversed the effect confirming that the delayed re-entry was because of the cilium [81]. These findings were further substantiated by reports that depletion of Tctex-1, a dynein light chain, resulted in

delayed cilia disassembly along with a delay in cell cycle re-entry in ciliated cells, but not in non-ciliated cells [69]. Recent advances in the understanding of link between cilia and cell cycle have revealed several mechanisms that connect cell cycle to positive and negative regulators of ciliogenesis, most of which are associated with the regulation of ciliary length as described in section 1.1.5.3 and figure 1.6. Thus, cilia appear to influence the crucial decision to divide or differentiate. These results point towards a suppressive role of cilia in cell cycle progression and suggest that presence of cilia provides a brake mechanism to restrict cell cycle progression in cancer cells. Also, from a therapeutic point of view, these results indicate that restoration of primary cilia in cancer cells could suppress the proliferation and growth of tumors.

With a perspective on tumorigenesis, a growing body of evidence suggests that the regulation of a normal cell cycle depends on the continuous tipping of balance to recruit the centrosome for either cell division or ciliogenesis. The primary cilium helps to keep cancer at bay by maintaining a balance in favor of normal cell growth. Any disturbance in this delicate balance may serve as a pro cancer stimulus and accelerate the progression of cell cycle. Misregulation of several cilia-associated proteins or shortening of ciliary length have been recognized as factors that can alter this balance in favor of tumorigenesis. Many proteins that are closely associated with ciliogenesis and cilia function have been implicated in cell cycle control. Malfunction of these cilia proteins result in cell cycle defects like alterations in cell cycle timing, fidelity of centrosome duplication, chromosome segregation and cytokinesis. Depletion of IFT88 in non-ciliated cells induces cell cycle progression whereas overexpression blocks G1/S transition [276]. Knockdown of IFT27 in Chlamydomonas delays cell cycle progression and affects cytokinesis [277]. It has also been shown that IFT27 and IFT46 are dynamically regulated during the cell cycle in Chlamydomonas, implying that transcriptional control of these IFT proteins is restricted within the cell cycle [278]. Ciliary protein BBS4 is required for the anchoring of microtubules to centrosome and has been shown to be essential for cell cycle progression [279]. Knockdown of BBS4 in cultured cells blocks the progression of cell division and induces apoptosis. Overall, these observations of cell cycle defects have led to the hypothesis that abnormally functioning cilia proteins disturb the balance between centrosome and basal body transition, leading to cell cycle checkpoint activation [111]. The disengagement of centrosomes from ciliation results in the activation of key regulators of G1/S transition, Cdk2 and cyclin E, which may tip the

balance towards tumorigenesis [111]. The above observations also provide evidence that many proteins initially linked to the cilium have functions that are not limited to the cilium alone, but are essential for cell cycle and other important cellular processes.

1.4. THE PRIMARY CILIUM AS A THERAPEUTIC TARGET

In spite of substantial progress in the identification of ciliopathy genes, no direct cilia therapies are available to the patients yet. Recent effort to treat a ciliopathy by gene therapy has yielded encouraging results. The utility of this approach was demonstrated in ORPK mice that were anosmic as a result of ciliary loss in their olfactory sensory neurons (OSN) due to mutations in IFT88 gene [280]. Rescue of ciliary structure and olfactory function was successfully achieved by adenoviral-mediated expression of IFT88 gene in fully differentiated OSNs of these mice. Thus, re-establishment of ciliary structure and function by gene therapy offers a viable therapeutic option for the treatment of ciliopathies. Pharmacological development of orphan drugs to specifically treat rare diseases like ciliopathies is economically unfeasible. Hence identification of cilia modulators from approved drugs already existing in the market could provide a quicker and economical solution for the treatment of ciliopathies. We have exploited this concept in our lab to identify a number of cilia modulating drugs, which is discussed in detail in the later part of this thesis. Presently, cilia proteome consists of up to 500 proteins, and this number is rising with the discovery of new ciliopathies and associated proteins [281, 282]. Since the biological functions of most of these proteins are unknown, the future approach should be aimed at performing genome or ciliome-wide screens to identify novel interactions of these proteins. Knowledge of these interactions will aid in the elucidation of new molecular mechanisms, which can be exploited to develop therapeutic strategies for ciliopathies in which, so far, only organ replacement therapy is the possible treatment option. Recently, bioengineering strategies are being employed to treat degenerative diseases by manipulating the length of primary cilia in stem cells [283]. Growing adult stem cells on nanotechnology implants with micro-grooved surfaces results in the disruption of biochemical pathway that regulates ciliary length. The manipulation of ciliary length leads to changes in stem cell differentiation, which can be controlled to alter the specialization of stem cells. Thus primary cilia can be used to control which specialized role stem cells go on to perform. This strategy is being considered to treat a number of degenerative conditions like Parkinson's disease, Alzheimer's disease and arthritis.

Several of the numerous proteins that are associated with ciliary structure or function might serve as new targets for knockdown and pharmacological inhibition studies to identify druggable targets. Since several transcription factors like RFX factors and FOXJ1 that regulate the transcriptional program in cilia formation have been implicated in the development of cancer [65], investigation of the signaling, structural and transcriptional aspects of the primary cilium has the potential to contribute valuable tools for improving the diagnosis and prognosis of cancers. A number of cilia-regulated signaling pathways implicated in oncogenesis (e.g. Hh pathway) are being considered as new cancer drug targets It must be noted that the efficacy of therapeutics targeting Hh signaling will rely on whether the activation of Hh pathway is dependent (upstream) or independent (downstream) of cilia. Thus, it can be challenging to manipulate an organelle that exerts both positive and negative effects on the pathway activity and tumorigenesis [284]. As a future direction, clinical relevance of the relationship between the presence of cilia and their responsiveness to Hh pathway inhibitors needs to be evaluated so that it can be used as a tool by clinicians to choose Hh-targeted drugs for treating individual cancers. From the perspective of diagnosis, presence of cilia can be exploited as a useful biomarker for the identification of Hh-dependent cancers that are responsive to Smo inhibitors. Since some types of human brain cancers possess cilia while others do not, cilia could also be the basis in diagnosing the cancer type. Additionally, primary cilium could serve as a useful diagnostic to direct the treatment by functioning as an indicator of the specific mutation responsible for a cancer and by identifying the aggressiveness of a tumor. Such strategies demonstrate the potential of primary cilium as a new avenue of treatment for cancers. Future studies should focus on understanding the relationship between cilium, cell cycle and ciliary signaling in order to facilitate the development of strategies that can regulate cancer and cell-division rates rather than targeting the cells themselves.

1.5. HYPOTHESIS

As detailed above, evidence is accumulating that the primary cilium functions as a unique cellular antenna that senses a number of external parameters and converts these to intracellular signals that regulate normal tissue development and homeostasis. Defects in the formation or functioning of the cilium contribute to various pathologies, ranging from classical ciliopathologies to cancer. In cancer the primary cilium seems to play a dual role in most cancer types like melanoma, breast, kidney, pancreatic,

colorectal, ovarian, and prostate cancers the primary cilium acts as a tumor-suppressing organelle and puts a brake on cell proliferation due to the common use of structural elements by both the cilium and the centrosome, required for chromosome segregation. Other specific cancer types like glioblastoma, basal cell carcinoma and medulloblastoma appear to be dependent on the signaling due to their dependence on signaling pathways, including Hh signaling, that is centralized in the primary cilium. Owing to these emerging roles of the primary cilium in cancer, we hypothesize that in most tumor types, restoration of the cilium might exert a therapeutic effect by reinstating the brake mechanism, thereby reducing the proliferative potential of cancer cells. On the contrary, in cancer types that are dependent on the presence of cilia, ablation of this organelle might produce an antineoplastic effect by attenuation of oncogenic signaling resulting in reduced cellular proliferation. Thus, modulation of the cilium may serve as a novel and attractive therapeutic strategy to treat different types of cancers. The identification and characterization of small molecules that have the ability to modulate ciliogenesis is essential to validate this hypothesis and to explore the therapeutic potential of this approach.

1.6. AIMS AND OBJECTIVES

In view of the central role of the primary cilium in cellular physiology and its emerging role in various diseases including cancer, the prime goal of this research project was to explore the feasibility of using small molecules to normalize the cilium in disease conditions, with cancer as the main paradigm. As currently only few chemical compounds affecting the cilium are known, the main aims of this project were:

I. To identify novel small molecule modulators of the primary cilium.
II. To explore the feasibility of these chemical modulators to normalize the cilium in cancer models (and a ciliopathy model).
III. To examine the mechanisms by which some of these compounds normalize ciliogenesis

To this end a small molecule screening platform was developed based on an IN Cell high content analysis screen involving immunostaining and quantification of ciliation in otherwise poorly ciliated pancreatic cancer cells. The feasibility of the identified hits to restore ciliogenesis in pancreatic cancer cells and to attenuate their proliferation was examined (Chapter 2).

For a selection of the compounds identified in Chapter 2, the role of secreted ATP and the purinergic receptor signaling pathway in cilium normalization was explored (Chapter 3).

In Chapter 4, the same screening platform was used to identify compounds from a kinase inhibitor library as cilium modulators.

Finally, an independent screening platform was set up based on SREBP1c-mediated cilium suppression in MDCK kidney cells. This platform was used to screen a library of new compounds. The identified compounds were tested in cancer models and in a ciliopathy model, and the involvement of vesicle trafficking in the normalization of the cilium was examined (Chapter 5).

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CHAPTER 2

Identification of Drugs that Restore Primary Cilium Expression in Cancer Cells

Identification of Drugs that Restore Primary Cilium Expression in Cancer Cells

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2.1. Abstract

The development of cancer is often accompanied by a loss of the primary cilium, a microtubule-based cellular protrusion that functions as a cellular antenna and that puts a break on cell proliferation. Hence, restoration of the primary cilium in cancer cells may represent a novel promising approach to attenuate tumor growth. Using a high content analysis-based approach we screened a library of clinically evaluated compounds and marketed drugs for their ability to restore primary cilium expression in pancreatic ductal cancer cells. A diverse set of 118 compounds stimulating cilium expression was identified. These included glucocorticoids, fibrates and other nuclear receptor modulators, neurotransmitter regulators, ion channel modulators, tyrosine kinase inhibitors, DNA gyrase/topoisomerase inhibitors, antibacterial compounds, protein inhibitors, microtubule modulators, and COX inhibitors. Certain compounds also dramatically affected the length of the cilium. For a selection of compounds (Clofibrate, Gefitinib, Sirolimus, Imexon and Dexamethasone) their ability to restore ciliogenesis was confirmed in a panel of human cancer cell line models representing different cancer

types (pancreas, lung, kidney, breast). Most compounds attenuated cell proliferation, at least in part through induction of the primary cilium, as demonstrated by cilium removal using chloral hydrate. These findings reveal that several commonly used drugs restore ciliogenesis in cancer cells, and warrant further investigation of their antineoplastic properties.

2.2. Introduction

The primary cilium is a single, microtubule-based structure that protrudes from the surface of most mammalian cells [1]. It functions as a cellular antenna that captures signals from the environment and serves as a hub of key developmental and homeostatic signaling pathways including Wnt, planar cell polarity, and Hedgehog signaling [2-4]. Defects that compromise ciliary function contribute to specific disorders including Polycystic Kidney Disease (PKD), Birt-Hogg-Dubé (BHD) syndrome, Bardet-Biedl Syndrome (BBS) and others. Interestingly, several of these syndromes predispose affected carriers to the onset of cancer [5-7]. Moreover, an ever-increasing number of papers report on a decrease, loss, or distortion of the primary cilium in a variety of cancer types [8-11]. These include pancreatic cancer, breast cancer, melanoma, and prostate cancer. It is commonly assumed that the cilium puts a break on cell proliferation as it uses the same structural components required for chromosome segregation [12-14]. Loss of the cilium in cancer cells may, therefore, release this break and, moreover, may contribute to distorted cellular signaling, which is a hallmark of cancer. Hence, restoration of the primary cilium in cancer cells may represent a novel promising approach to attenuate cell proliferation and may provide novel opportunities for therapeutic antineoplastic intervention. However, so far few chemical compounds are known that normalize ciliogenesis in cancer cells.

Here, we have established a high content analysis-based screening method to identify small molecules that have the ability to restore the primary cilium in cancer cells. Further application of this method to screen a repurposing library composed of clinically evaluated compounds and marketed drugs revealed that many commonly used drugs restore the primary cilium in cancer cells and attenuate cell proliferation. These findings provide new insight into the spectrum of action of some commonly used drugs and may promote the expedited application of cilia-based therapies via repurposing of existing drugs in the field of oncology and beyond.

2.3. Results

2.3.1. High content analysis-based screening for cilium-inducing compounds in pancreatic ductal cancer cells

To screen a compound library for potential modulators of ciliogenesis, we developed an immunofluorescence microscopy-based phenotypic screening strategy in a 96-well format using an IN Cell 2000 High Content Analyzer (Figure 2.1). Pancreatic ductal cancer cells were chosen as the prime biological model in view of their well-documented loss of the primary cilium [10]. From a preliminary screening of a panel of human pancreatic cancer cell lines, CFPAC-1 cells were selected for the compound screening based on their ability to grow as flat monolayers, which is a prerequisite for accurate automated image acquisition, and for their inherent low rate of ciliogenesis, even at a high confluence (Figure 2.2). After incubation with 1600 different compounds from the Pharmakon 1600 library (all drugs added at 10 micromolar in DMSO), the percentage of ciliated cells was assessed in single wells by fluorescence microscopy-based visualization of the primary cilium using antibodies targeting cilium-associated acetylated tubulin. Nuclei were stained with Hoechst-33258. Images were analyzed using IN Cell Developer software according to the workflow illustrated in Figure 2.2. A cell was considered ciliated when the primary cilium signal (fluorescent dot) was enclosed within the nuclear border of the segmented nuclei. If more than one cilium-like dot was detected within the nuclear border, the bigger dot was selected as the match. The number of ciliated cells was then determined by counting the total number of nuclei and linked cilia in 20 fields in a single well. Compounds were considered ciliogenic based on their ability to increase the percentage of ciliated cells by at least 3 standard deviations compared to vehicle control. Using this approach, 156 ciliogenic compounds were identified in the initial screen. To eliminate false positives, the initial hits were re-evaluated in triplicate in a secondary screen using the same screening strategy. In this screen 118 cilium-enhancing compounds were confirmed. 110 of these compounds were found to increase ciliogenesis by at least a factor 2 (Figure 2.3A). Besides cilium-enhancing compounds, we identified 22 compounds that decreased ciliogenesis by at least 2-folds. The stimulatory compounds were categorized into 9 different classes based on their potential molecular targets (Table 2.1). More than one third of the positive compounds (49/118) were classified as glucocorticoids, fibrates or other nuclear receptor modulators (Figure 2.3B). 14/118 compounds were categorized as neurotransmitter modulators. Other classes included ion channel modulators, tyrosine

kinase inhibitors, DNA gyrase/topoisomerase inhibitors, antibacterial compounds, protein synthesis inhibitors, microtubule modulators, and COX inhibitors.

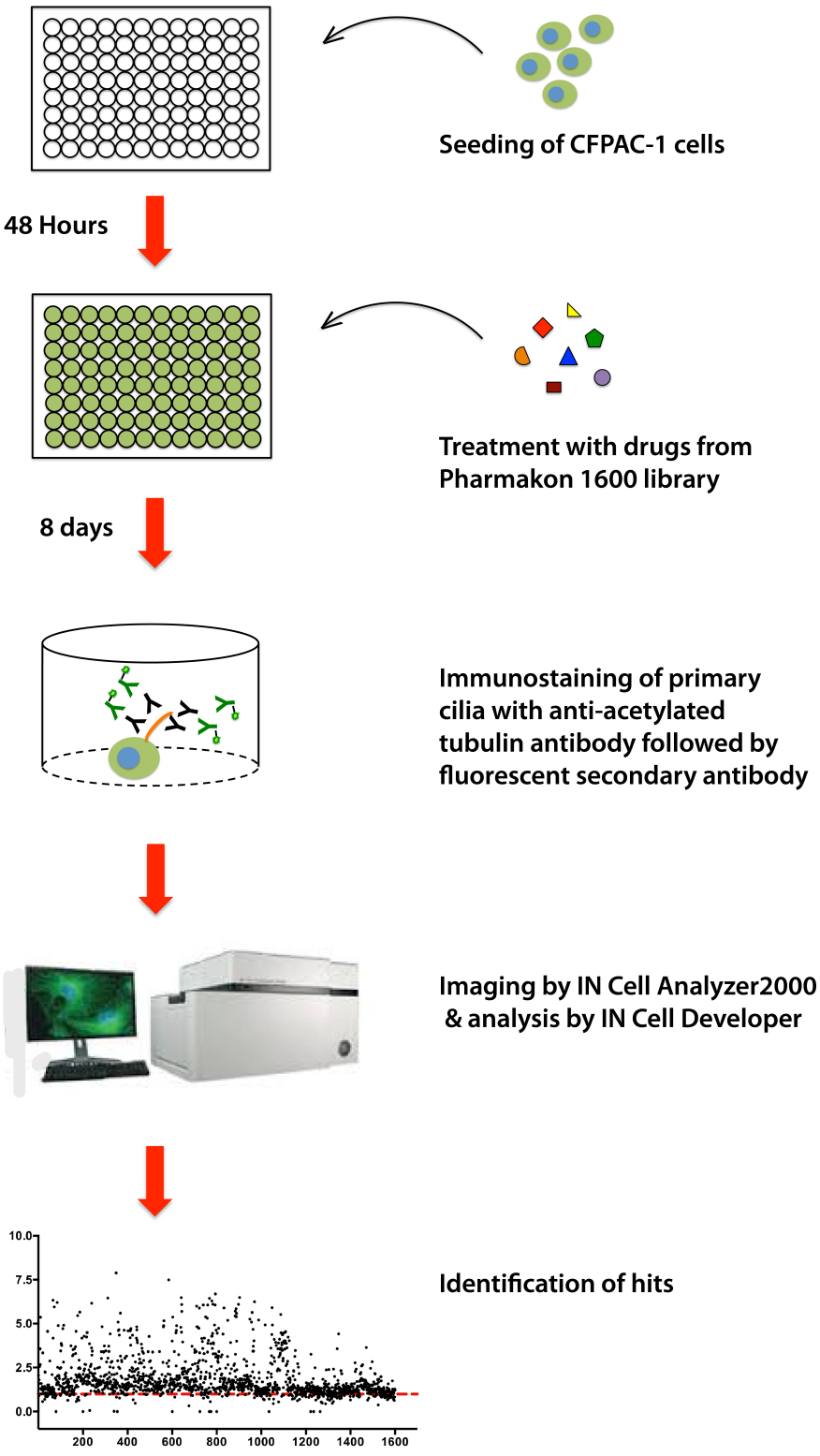

Figure 2.1. Schematic representation of the screening strategy of the Pharmakon Library using the human CFPAC-1 pancreatic cancer cell line model.

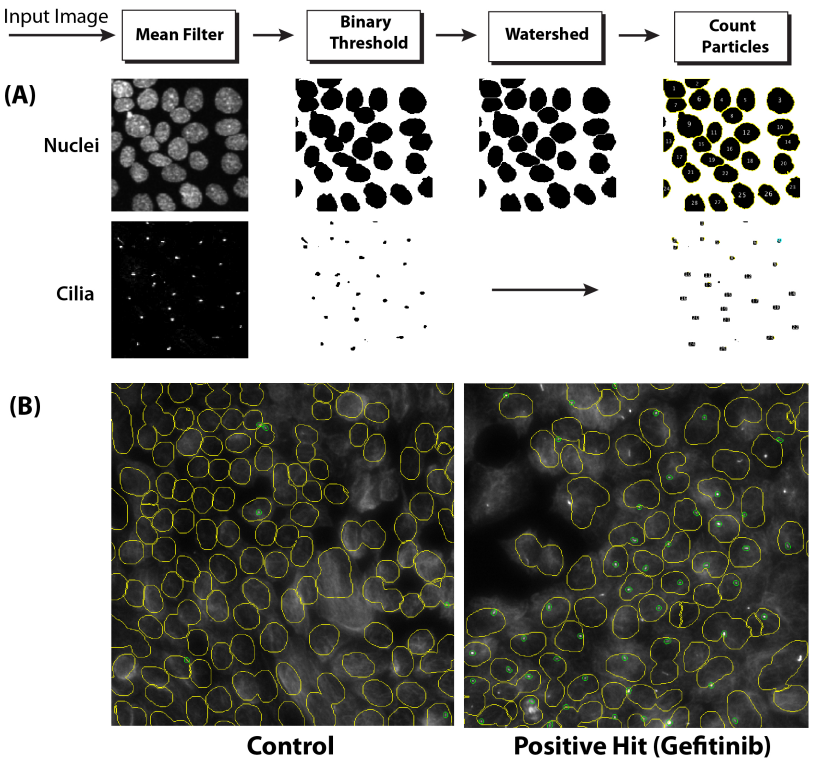

Figure 2.2. Imaging of ciliated cells using the IN Cell Analyzer 2000 Imaging system. (A) Depiction of the image processing procedure, including the use of a watershed clump breaking algorithm to delineate nuclei. (B) Example of processed IN Cell Analyzer images of poorly ciliated control CFPAC-1 cells (left panel) and well-ciliated Gefitinib-treated CFPAC-1 cells (right panel). Nuclei are delineated by yellow lines. Cilia are indicated by small green circles.

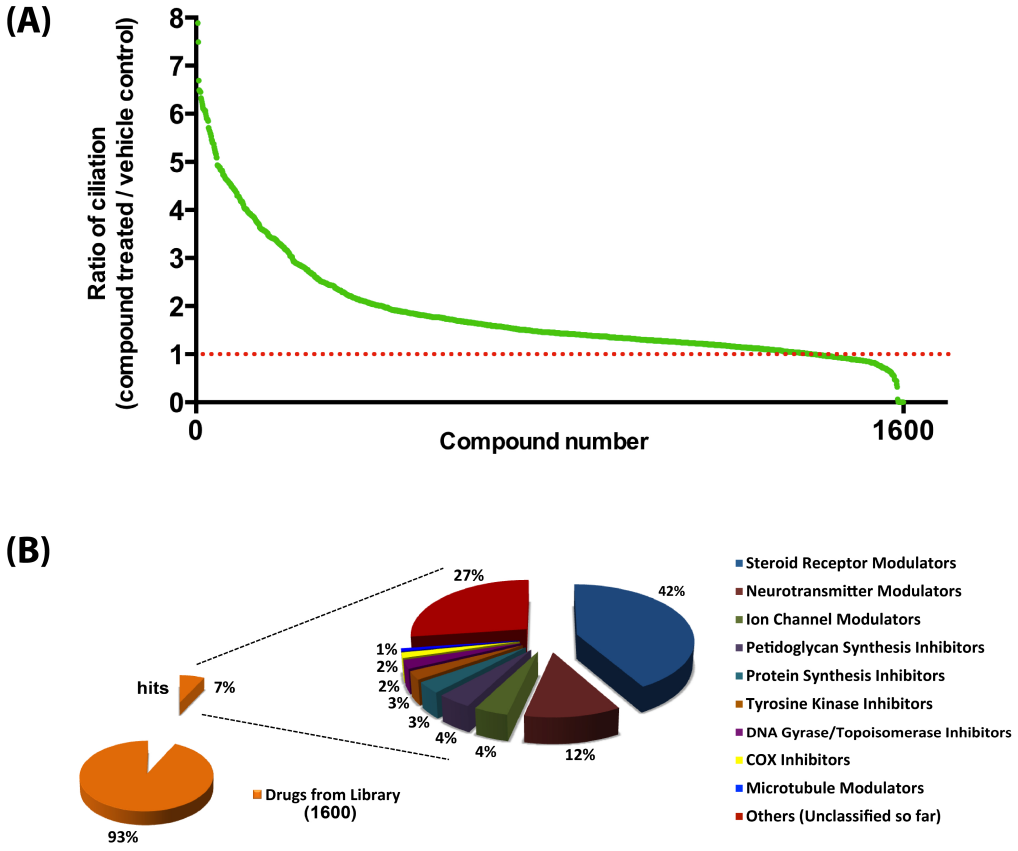

Figure 2.3. Summary of the outcome of the high content screen. (A) Ciliogenic capacity of 1600 compounds of the Pharmakon 1600 library in CFPAC-1 cells using IN Cell Analyzer high content analysis. Compounds are ranked according to their potency to increase the percentage of ciliated cells relative to vehicle-treated cells (red dotted line). (B) Target diversity of confirmed hits is shown as a percentage of abundances of compounds in each class.

Table 2.1. List of ciliogenic drugs identified from the Pharmakon 1600 library screen. Compounds are classified according to their potential targets and arranged in descending order of ciliogenic potential in CFPAC-1 cells as assessed by IN Cell analysis (expressed as ratio of % of ciliated cells in treated cultures versus vehicle control) within each group. GR, glucocorticoid receptor; MR, mineralocorticoid receptor; ER, estrogen receptor; PR, progesterone receptor; THR, thyroid hormone receptor; MAO, mono amine oxidase; DR, dopamine receptor; NCR, nicotinic cholinergic receptor; MOR, mu-opioid receptor; A1AR, alpha 1 adrenergic receptor; A2AR, alpha 2 adrenergic receptor; B1AR, beta 1 adrenergic receptor; COX, cyclooxygenase; TKR, tyrosine kinase receptor; ACE, angiotensin-converting enzyme.

DRUG NAME	CILIATION RATIO	ACTION	TARGET
1. STEROID RECEPTOR MODULATORS			
Glucocorticoid receptor modulators			
HYDROCORTISONE BUTYRATE	6.33	Glucocorticoid, anti-inflammatory	GR
AMCINONIDE	6.30	Glucocorticoid, anti-inflammatory	GR
DESONIDE	6.25	Anti-inflammatory, glucocorticoid	GR
CLOBETASOL PROPIONATE	6.11	Glucocorticoid, anti-inflammatory	GR
PREDNISOLONE HEMISUCCINATE	6.10	Anti-inflammatory, glucocorticoid	GR
BETAMETHASONE 17,21-DIPROPIONATE	6.00	Glucocorticoid, anti-inflammatory	GR
ALCLOMETAZONE DIPROPIONATE	5.89	Anti-inflammatory, glucocorticoid	GR
DESOXYMETASONE	5.64	Anti-inflammatory	GR
BETAMETHASONE ACETATE	5.26	Anti-inflammatory	GR
PREDNICARBATE	5.19	Anti-inflammatory, glucocorticoid	GR
TRIAMCINOLONE DIACETATE	5.16	Anti-inflammatory	GR
DEXAMETHASONE	5.09	Glucocorticoid	GR
PREDNISOLONE SODIUM PHOSPHATE	5.09	Anti-inflammatory, glucocorticoid	GR
METHYLPREDNISOLONE SODIUM SUCCINATE	4.93	Glucocorticoid, anti-inflammatory	GR
TRIAMCINOLONE ACETONIDE	4.88	Anti-inflammatory	GR
BECLOMETHASONE DIPROPIONATE	4.83	Anti-asthmatic, topical antiinflammatory	GR
TRIAMCINOLONE	4.82	Glucocorticoid	GR
HYDROCORTISONE HEMISUCCINATE	4.82	Glucocorticoid	GR
FLUOROMETHOLONE	4.71	Glucocorticoid, Anti-inflammatory	GR
ISOFLUPREDNONE ACETATE	4.68	Anti-inflammatory	GR
DEXAMETHASONE SODIUM PHOSPHATE	4.66	Glucocorticoid, Anti-inflammatory	GR
FLUOCINOLONE ACETONIDE	4.65	Glucocorticoid, Anti-inflammatory	GR
HYDROCORTISONE PHOSPHATE TRIETHYLAMINE	4.64	Glucocorticoid	GR
DEFLAZACORT	4.62	Anti-inflammatory	GR
BUDESONIDE	4.61	Anti-inflammatory	GR
DEXAMETHASONE ACETATE	4.60	Glucocorticoid, Anti-inflammatory	GR
FLUMETHAZONE PIVALATE	4.59	Glucocorticoid, Anti-inflammatory	GR
PREDNISOLONE ACETATE	4.28	Glucocorticoid	GR
FLUDROCORTISONE ACETATE	4.20	Mineralocorticoid	GR/MR
PREDNISOLONE	4.27	Glucocorticoid	GR
HYDROCORTISONE ACETATE	4.18	Glucocorticoid, antiinflammatory	GR
FLUTICASONE PROPIONATE	4.12	Anti-inflammatory	GR
FLUNISOLIDE	3.87	Anti-inflammatory	GR
FLUMETHASONE	3.82	Anti-inflammatory	GR
FLUOCINONIDE	3.78	Anti-inflammatory, Glucocorticoid	GR/MR
DIFLORASONE DIACETATE	3.74	Anti-inflammatory, glucocorticoid	GR
BETAMETHASONE SODIUM PHOSPHATE	3.62	Anti-inflammatory, glucocorticoid	GR
BETAMETHASONE VALERATE	3.56	Glucocorticoid	GR
MEDRYSONE	3.54	Glucocorticoid	GR
HYDROCORTISONE VALERATE	3.52	Anti-inflammatory, glucocorticoid	GR
FLURANDRENOLIDE	3.45	Anti-inflammatory	GR
BETAMETHASONE	3.40	Glucocorticoid, anti-inflammatory	GR
METHYLPREDNISOLONE	3.34	Glucocorticoid	GR
Estrogen receptor modulators			
ESTRADIOL BENZOATE	3.96	Estrogen	ER
Progesteron receptor modulators			
MEDROXYPROGESTERONE ACETATE	3.42	Contraceptive	PR agonist
Thyroid receptor modulators			
IOPANIC ACID	3.29	Radio-opaque agent	5'deiodinase 73-74 conversion
LIOTHYRONINE	1.35	Thyroid hormone blocker, antidepressant	THR

PPAR receptor modulators			
CLOFIBRATE	6.06	Antihyperlipidemic	(PPAR) alpha agonist
PIOGLITAZONE HYDROCHLORIDE	2.15	Antidiabetic	(PPAR) gamma agonist
2. NEUROTRANSMITTER MODULATORS			
Serotonin transporter inhibitors			
PAROXETINE HYDROCHLORIDE	6.69	Antidepressant	Serotonin transporter
MILNACIPRAN HYDROCHLORIDE	3.80	Inhibitor of norepinephrine and seritonin uptake, treatment of fibromyalgia	Serotonin transporter
Serotonin breakdown inhibitors			
PARGYLINE HYDROCHLORIDE	4.55	Antihypertensive	MAO
Serotonin receptor inhibitors			
TEGASEROD MALEATE	5.90	5HT4 receptor agonist, peristaltic stimulant	5HT receptor
ALMOTRIPTAN	3.96	5HT 1B/2D receptor agonist	5HT receptor
RIZATRIPTAN BENZOATE	1.45	5HT-1B/1D agonist, antimigraine	5HT receptor
Dopamine receptor modulators			
PERGOLIDE MESYLATE	3.59	Dopamine receptor agonist	DR
Nicotinic cholinergic receptor modulators			
GALLAMINE TRIETHIODIDE	2.27	Muscle relaxant (skeletal)	NCR antagonist
Mu-opiod receptor modulators			
LOPERAMIDE HYDROCHLORIDE	3.85	Ca channel blocker	MOR agonist
Alpha1-adrenergic receptor modulators			
OXYMETAZOLINE HYDROCHLORIDE	3.55	Adrenergic agonist, nasal decongestant	A1AR agonist
ADRENOLONE HYDROCHLORIDE	2.42	Adrenergic (opthalmic)	A1AR agonist
Alpha2-adrenergic receptor modulators			
XYLAZINE	3.76	Analgesic	A2AR
Beta1-adrenergic receptor modulators			
DOBUTAMINE HYDROCHLORIDE	4.83	Cardiotonic	B1AR agonist
ACEBUTOLOL HYDROCHLORIDE	3.90	Antihypertensive, antianginal, antiarrhythmic	B1AR antagonist
3. ION CHANNEL MODULATORS			
CAPSAICIN	4.52	Analgesic (topical)	TRPV1 channel
ESOMEPRAZOLE POTASSIUM	4.47	Gastric acid secretion inhibitor	H+/K+ exchange, alpha polypeptide
BUPIVACAINE HYDROCHLORIDE	4.29	Anesthetic (local)	SCN10A blocker
SURAMIN HEXASODIUM	2.85	Antiprotozoal, trypanocidal, antiviral	ATP-activated ion channel blocker
OXCARBAZEPINE	1.38	Antipsychotic / Na channel inhibition	SCN1A blocker
4. COX INHIBITORS			
KETOROLAC TROMETHAMINE	3.92	Anti-inflammatory / cyclooxygenase	COX
INDOPROFEN	1.88	Analgesic, anti-inflammatory / cyclooxygenase	COX
5. TYROSINE KINASE INHIBITORS			
GEFITINIB	4.89	Antineoplastic / EGFR inhibitor	TKR
LEFLUNOMIDE	4.24	Antineoplastic, PDGF receptor blocker	TKR
TANDUTINIB	2.81	Tyrosine kinase inhibitor	TKR
6. ANTIBACTERIAL PEPTIDOGLYCAN SYNTHESIS INHIBITORS			
CEFPROZIL	4.48	Antibacterial	Peptidoglycan synthesis
CEFOXITIN SODIUM	4.11	Antibacterial	Peptidoglycan synthesis
CEFAMANDOLE SODIUM	3.76	Antibacterial	Peptidoglycan synthesis
HETACILLIN POTASSIUM	1.90	Antibacterial	Peptidoglycan synthesis
CEFTIBUTEN	1.42	Antibacterial	Peptidoglycan synthesis
7. DNA GYRASE/TOPOISOMERASE INHIBITORS			
CIPROFLOXACIN	6.45	Antibacterial, fungicide	DNA gyrase topoisomerase
GEMIFLOXACIN MESYLATE	4.49	Antibacterial	DNA gyrase topoisomerase
LOMEFLOXACIN HYDROCHLORIDE	3.94	Antibacterial	DNA gyrase topoisomerase
8. PROTEIN SYNTHESIS INHIBITORS			
SIROLIMUS	6.06	Immunosuppressant, antineoplastic; rapamycin	mTOR / protein synthesis
OXYTETRACYCLINE	4.02	Antibacterial	Protein synthesis
MECLOCYCLINE SULFOSALICYLATE	3.57	Antibacterial	Protein synthesis
GENTAMICIN SULFATE	2.45	Antibacterial	Protein synthesis
9. MICROTUBULE MODULATORS			
DOCETAXEL	2.51	Antineoplastic	Microtubule

OTHERS (unclassified so far)			
IMEXON	5.39	Antineoplastic	
SEMUSTINE	5.31	Antineoplastic	
DENATONIUM BENZOATE	4.92	Denaturing agent, bitter principle	
TRANILAST	4.82	Antiallergic, mast cell degranulation inhibitor, angiogenesis blocker	
DICHLORISONE ACETATE	4.42	Antipruretic	
OSELTAMIVIR PHOSPHATE	4.38	Antiviral	Neuraminidase
DIBENZOTHIOPHENE	4.36	Keratolytic	
PENTAGASTRIN	4.29	Gastric secretion indicator	
RETINYL PALMITATE	4.05	Provitamin, antixerophthalamic	
SULCONAZOLE NITRATE	4.03	Antifungal	Sterol 14alpha-demethylase
DOCUSATE SODIUM	4.02	Stool softener	Anionic surfactant
BIFONAZOLE	3.93	Antifungal, calmodulin antagonist	Sterol 14alpha-demethylase
TRANDOLAPRIL	3.89	Antihypertensive, ACE inhibitor	ACE
PROCARBAZINE HYDROCHLORIDE	3.60	Antineoplastic	
MANNITOL	3.59	Diuretic, sweetener, diagnostic aid	
ARGININE HYDROCHLORIDE	3.32	Ammonia detoxicant, diagnostic aid	
TOLNAFTATE	3.31	Antifungal	Squalene epoxidase
PENTETIC ACID	3.18	Chelating agent, diagnostic aid	
AVOBENZONE	3.04	Sunscreen	
IODOQUINOL	2.99	Antiamebic	
PRILOCAINE HYDROCHLORIDE	2.93	Anesthetic (local)	
AMPYZINE SULFATE	2.88	CNS stimulant	
BENZOXIQUINE	2.71	Anti-infective	
PRASUGREL	2.69	Platelet aggregation inhibitor	
TRIENTINE HYDROCHLORIDE	2.60	Chelating agent	
PARAROSANILINE PAMOATE	2.56	Antihelminthic, antischistosomal	
NADIDE	2.24	Alcohol and narcotic antagonist	
ANEBROMPHENIRAMINE MALEATE	2.14	H1 antihistamine	
ISOTRETINON	2.12	Anti-acne, antineoplastic	
ANETHOLE	2.02	Expectorant, gastric stimulant, insecticide	
CLOFZAMINE	1.92	Antibacterial, antilepretic, antituberculosis	Guanine, PLA2, inhibits Smase
LITHIUM CITRATE	1.38	Antidepressant	

2.3.2. Confirmation of ciliogenic ability of representative compounds by confocal microscopy.

From the 118 cilium-enhancing compounds identified in the IN Cell Analyzer screen, we selected 20 representative compounds from the different classes for reconfirmation of their cilium modulating effect by a more robust confocal fluorescence microscopy approach, which also allowed assessment of changes in cilium length (Figure 2.4). Clofibrate, Gefitinib, Sirolimus, Imexon, Ciprofloxacin, and Dexamethasone were the most potent inducers of cilia in terms of percentage of ciliated cells, showing a statistically significant increase of 4-fold or more, relative to control vehicle-treated cells (Figure 2.4A). In terms of cilium length, Imexon, Clofibrate, and Xylazine induced a significant increase of 4-fold or more, relative to control conditions (Figure 2.4B). Some compounds like Clofibrate and Imexon were potent inducers of both cilium percentage and cilium length whereas other compounds primarily enhanced cilium percentage (e.g. Gefitinib). Figure 2.5A shows representative confocal microscopy pictures of primary ciliation of cells treated with the most potent compounds as revealed by staining for

acetylated tubulin. To confirm that genuine primary cilia are induced by the drugs, cilia were stained for IFT88, an alternative marker of the primary cilium (Figure 2.5B).

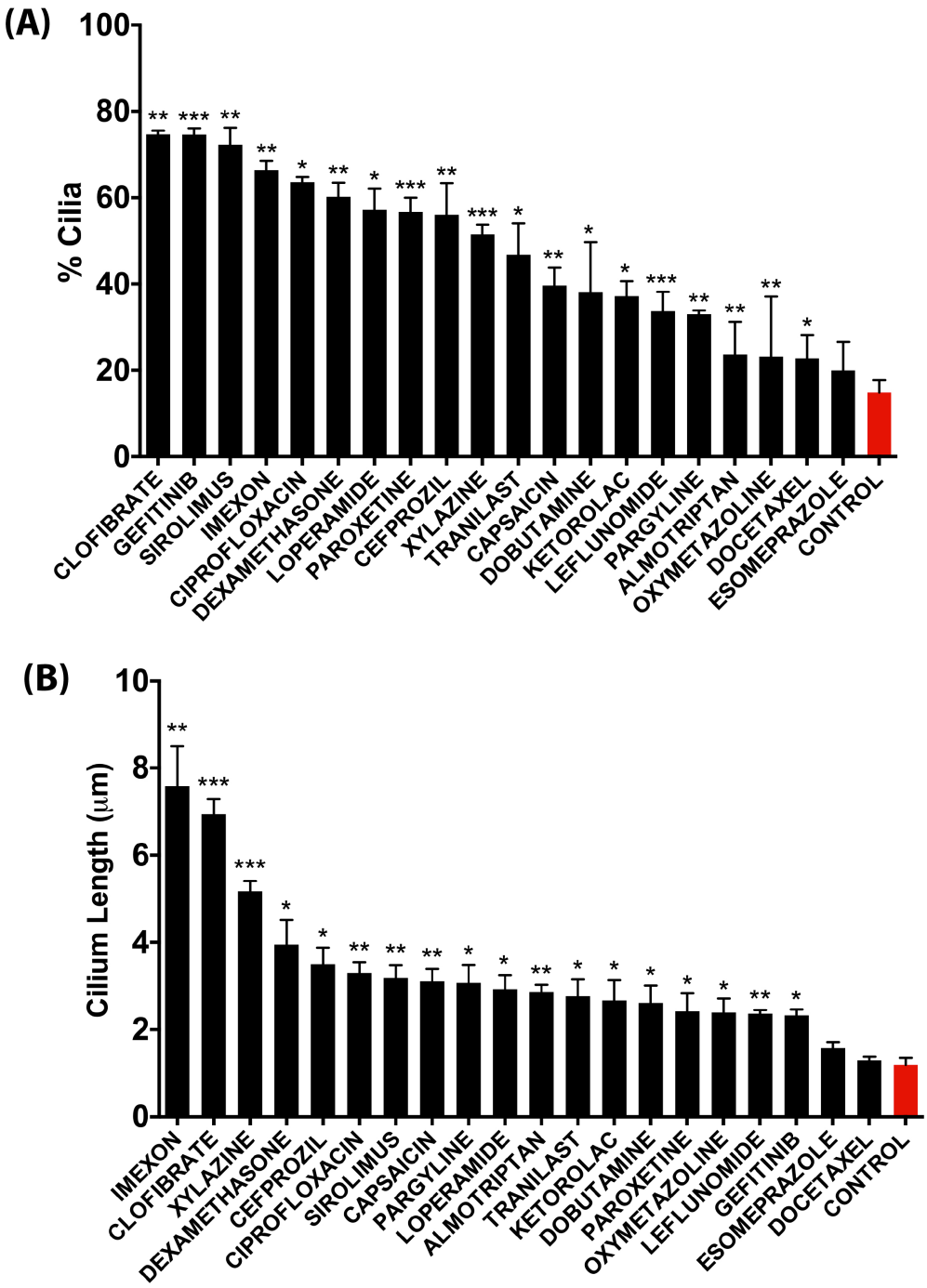

Figure 2.4. Quantitative analysis of the effect of representative compounds on the percentage of ciliation (A) and on average cilium length (B) in CFPAC-1 cells as assessed by confocal fluorescence microscopy analysis. Quantification was performed by counting 100 - 300 cells from at least three regions of the well. Data are presented as mean ±SEM, *p ≤ 0.05, **p ≤ 0.005, ***p ≤ 0.0005 as compared to control.

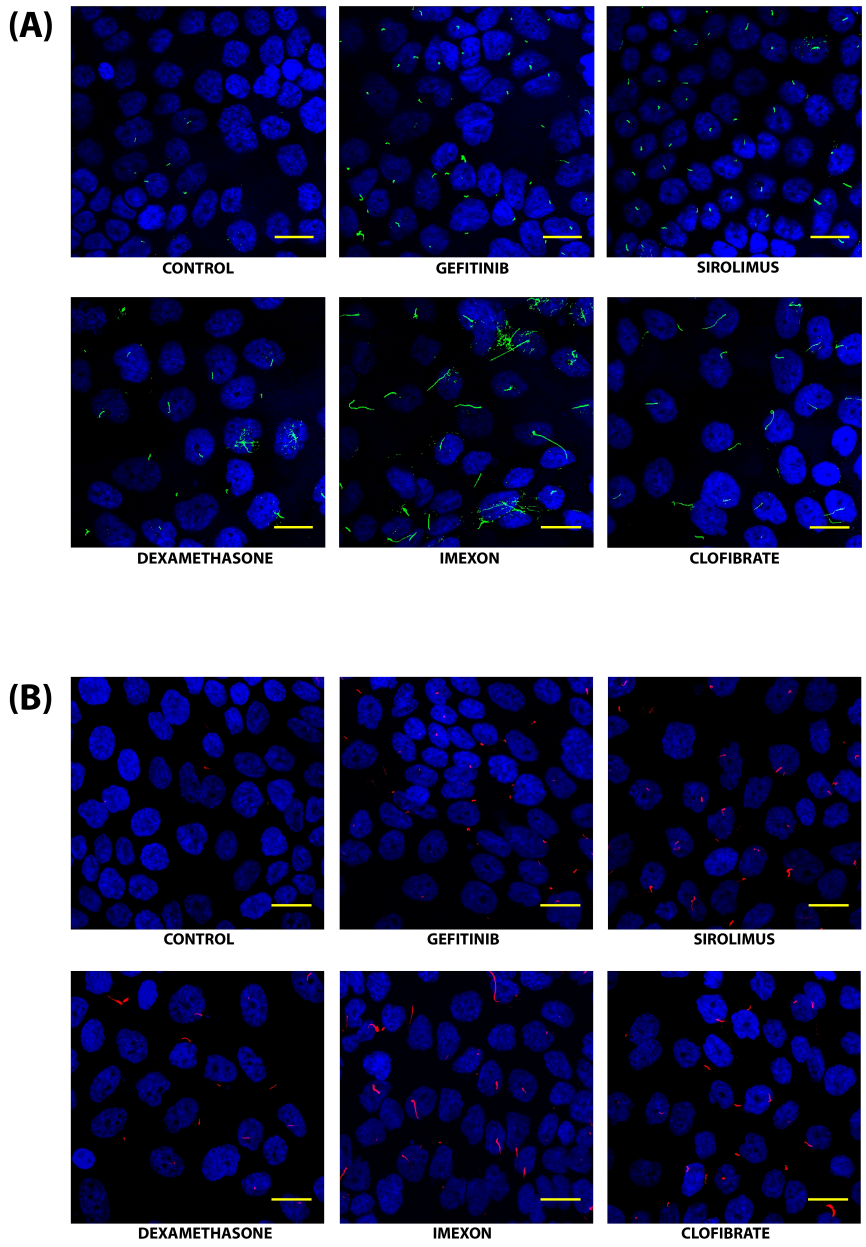

Figure 2.5. Confocal fluorescence microscopy images of primary cilia in CFPAC-1 cells treated with selected compounds. Cilia were stained with an antibody against acetylated tubulin (green) (A) or with an antibody against IFT88 (red) (B). Nuclei were visualized by staining with DAPI (Blue). Images were captured using Bio-Rad Radiance confocal microscope through a 40X objective lens at 2.3X zoom. The scale bar represents 20 μm. Images were processed manually to optimally visualize cilia.

2.3.3. Identified ciliogenic drugs induce cilia in multiple cancer cell models

To corroborate the ability of these compounds to induce cilia in cancer cells, we tested a selection of the most potent compounds (Clofibrate, Gefitinib, Sirolimus, Imexon and Dexamethasone) in a panel of human cell lines representing different cancer types: A549 (lung cancer), UMRC2 (kidney cancer), SUM159 (breast cancer) and L3.6 (pancreatic cancer) cell lines. As shown in Figure 2.6, all compounds significantly increased the

percentage of ciliated cells in all the four cell lines. These results confirm the potential of the identified compounds as cilium inducers in cancer cells.

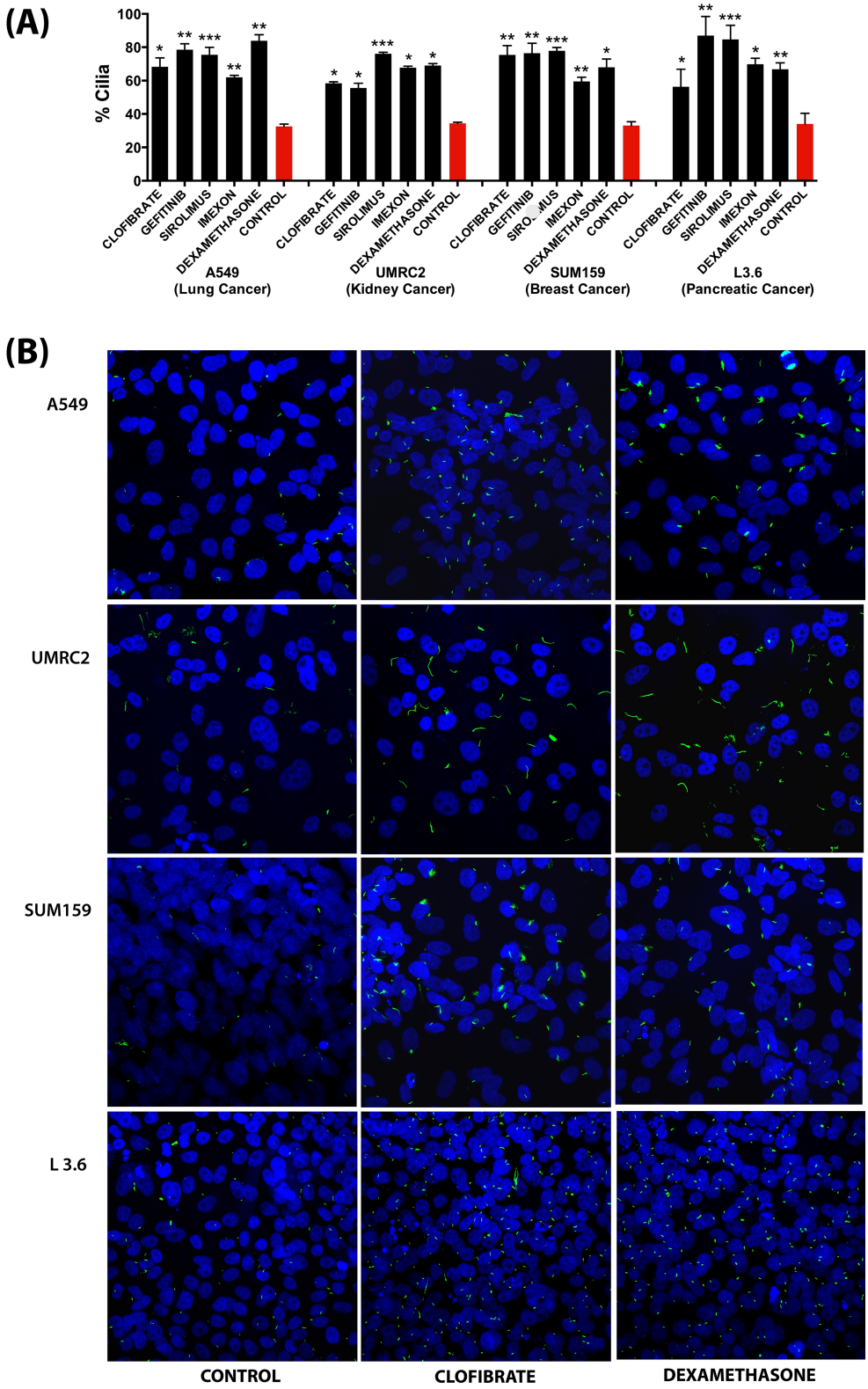

Figure 2.6. Effect of a selection of compounds on ciliogenesis in different cancer cell line models as assessed by confocal fluorescence microscopy analysis. (A) Quantification of the percentage of ciliated cells. Data are presented as mean (n = 100-300) ±SEM, *p ≤ 0.05, **p ≤ 0.005, ***p ≤ 0.0005 as compared to control. (B) Representative images showing the effect of selected compounds on ciliation in different cancer cell line models. All Images were captured using Nikon C2 Eclipse Ti-E confocal microscope at 1.0X zoom using a 60x objective lens.

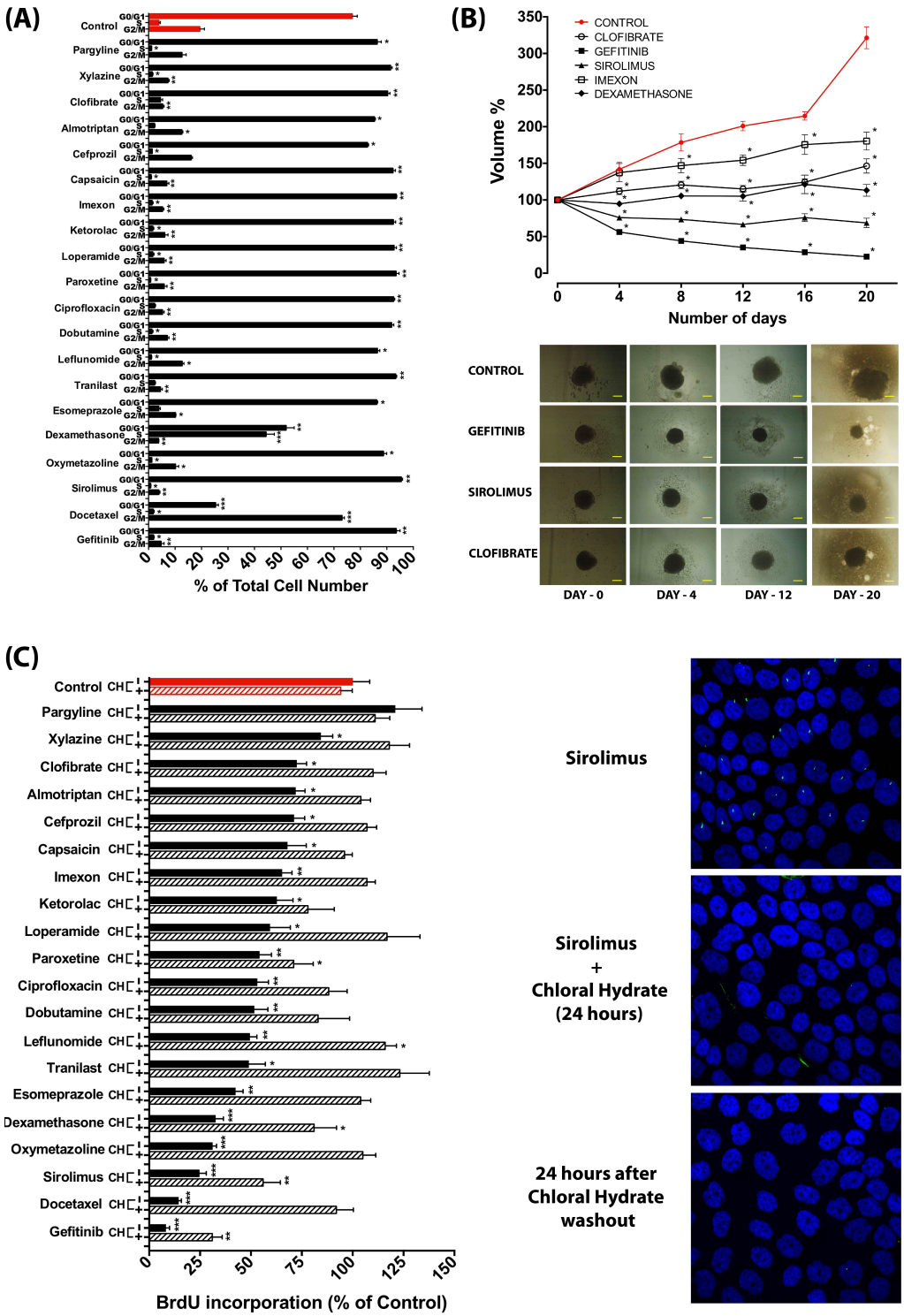

Figure 2.7. Anti-proliferative effect of ciliogenic compounds and involvement of the primary cilium. (A) Changes in cell cycle profile as determined by FACS analysis of CFPAC-1 cells upon treatment with a selection of ciliogenic compounds. Data are presented as mean ±SEM, *p ≤ 0.05, **p ≤ 0.005, ***p ≤ 0.0005 as compared to control. (B) Effect of selected compounds on spheroid formation of L3.6 pancreatic cancer cells. Data are presented as mean of at least 6 spheroids ±SEM, *p < 0.0001 as compared to control. The bottom panel shows representative images of spheroids treated with vehicle control and with selected drugs. (C) Effect of compounds on cell proliferation of CFPAC-1 cells as measured by BrdU incorporation and impact of deciliation by treatment with chloral hydrate (CH). Proliferation of cells was measured by BrdU incorporation 24 hours after deciliation. Differences in proliferation were expressed as percentage of BrdU incorporation as compared to untreated control (red bar). Data are presented as mean ±SEM, *p ≤ 0.05, **p ≤ 0.005, ***p ≤ 0.0005.Confocal microscope images show the effect of 4 mM chloral hydrate on sirolimus-treated CFPAC-1 cells. Primary cilia are stained for acetylated tubulin (green) and nuclei are stained with DAPI (blue).

2.3.4. Ciliogenic drugs attenuate cell proliferation at least in part through induction of the primary cilium

As the presence of the primary cilium is dependent on the cell cycle and is most prominent in the G0/G1 phase, we examined the effect of the selected drugs on the cell cycle using FACS analysis. Although under the culture condition that we used, cultures were not highly proliferative even in control conditions, most compounds resulted in a further increase in the percentage of cells in the G0/G1 phase, indicative of a further induction of growth arrest (Figure 2.7A). In line with these findings, most compounds attenuated cell proliferation as assessed by a spheroid formation assay of L3.6 cells (Figure 2.7B) and BrdU incorporation in CFPAC-1 cells (Figure 7C). To explore to what extent primary cilium occurs secondarily to the growth arrest or in fact actively contributes to the observed attenuation of cell proliferation, we assessed the effect of these compounds on cell proliferation in the presence of the deciliation agent chloral hydrate, which completely removed the cilium (Figure 2.7C). Interestingly, in most cases deciliation largely restored cell proliferation of compound-treated cells (Figure 2.7C), indicating that the antiproliferative effect of these compounds is at least in part caused by their ability to induce the primary cilium.

2.4. Discussion

Building on the emerging concept that loss of the primary cilium is linked to the development of several tumor types and that re-establishing the expression of this organelle may attenuate tumor growth, we have developed a semi-high-throughput approach to identify stimulators of ciliogenesis in cancer cells. Screening of the Pharmakon 1600 library resulted in the identification of 118 compounds that showed the ability to restore cilium expression in cancer cell line models. Among these were many glucocorticoids (43/118 hits or 43/47 glucocorticoids in the entire library). A diverse set of other drugs was identified including many neurotransmitter regulators and ion channel modulators. Some of the compounds from our screen such as dexamethasone, hydrocortisone, estradiol, pargyline and lithium have previously been described as enhancers of the percentage of ciliated cells and/or of cilium length [15-19]. Others, like beclomethasone, fluticasone, flunisolide, mannitol and arginine are known to affect ciliary beat frequency (CBF), which in many cases varies with dosage and length of treatment [20-23]. It has been suggested that beta-adrenergic receptor modulators may have a therapeutic role in the treatment of Primary Cilia Dyskinesia

(PCD) [24, 25]. Pioglitazone has been shown to have beneficial effects in the treatment of Polycystic Kidney Disease (PKD) [26]. Cilio-modulatory activities have also been attributed to several other compounds from our screen like triamcinolone, budesonide, paroxetine, oxymetazoline and docetaxel [23, 27-30]. Imexon, a potent enhancer of cilium length as shown in our results, is a known inducer of apoptosis and cell cycle arrest in pancreatic cancer cells [31]. For this reason it has been used in combination drug trials along with gemcitabine for the treatment of patients with advanced pancreatic cancer. The anti-diarrheal drug loperamide, a good inducer of cilia in our models, has been reported as a potential anti-tumor agent due to its ability to induce apoptosis [32].

With respect to the mechanisms underlying the cilium modulating effects of these compounds, many of them (steroid receptor modulators, neurotransmitter modulators, ion channel modulators) are known to affect levels of cAMP, calcium or other ions, which are established regulators of cilium expression and/or cilium length [33-35] (Figure 2.8). Microtubule modulators may affect the microtubule assembly and extension in the cilium or may disturb the transport towards the cilium [36-38]. Tyrosine kinase inhibitors may enhance ciliogenesis by inhibiting ligand-dependent activation of cell-cycle entry or by regulating the coordination of signaling events linked to cilium-centrosome axis, that control cell cycle, differentiation and migration [39, 40]. Ciliogenic effects of sirolimus (rapamycin) might be mediated through mTOR signaling which is known to modulate ciliary size and function through translational regulation [41]. Others (cephalosporins, DNA gyrase/topoisomerase inhibitors) are known to affect Aurora kinases [42-44], which are also well-established regulators of the primary cilium [45-47]. Several compounds may affect the cilium through extracellular ATP. Several studies show a relationship between intracellular calcium levels and extracellular ATP via the purinergic receptor [48-51]. Also glucocorticoids promote ATP release from the cell [52]. Many of these compounds also affect cell proliferation as known from the literature [31, 53-63] or as revealed in our spheroid and BrdU assays. This effect was not always proportional to the induction of ciliogenesis, which is not surprising given the various mechanisms of action of these compounds. The further characterization of these mechanisms may be challenging in part because of the incomplete understanding of the mechanisms of action of some of these drugs and the processes involved in ciliogenesis. Many of the compounds we identified cause cell cycle arrest in the G0/G1 phase, which is known to promote

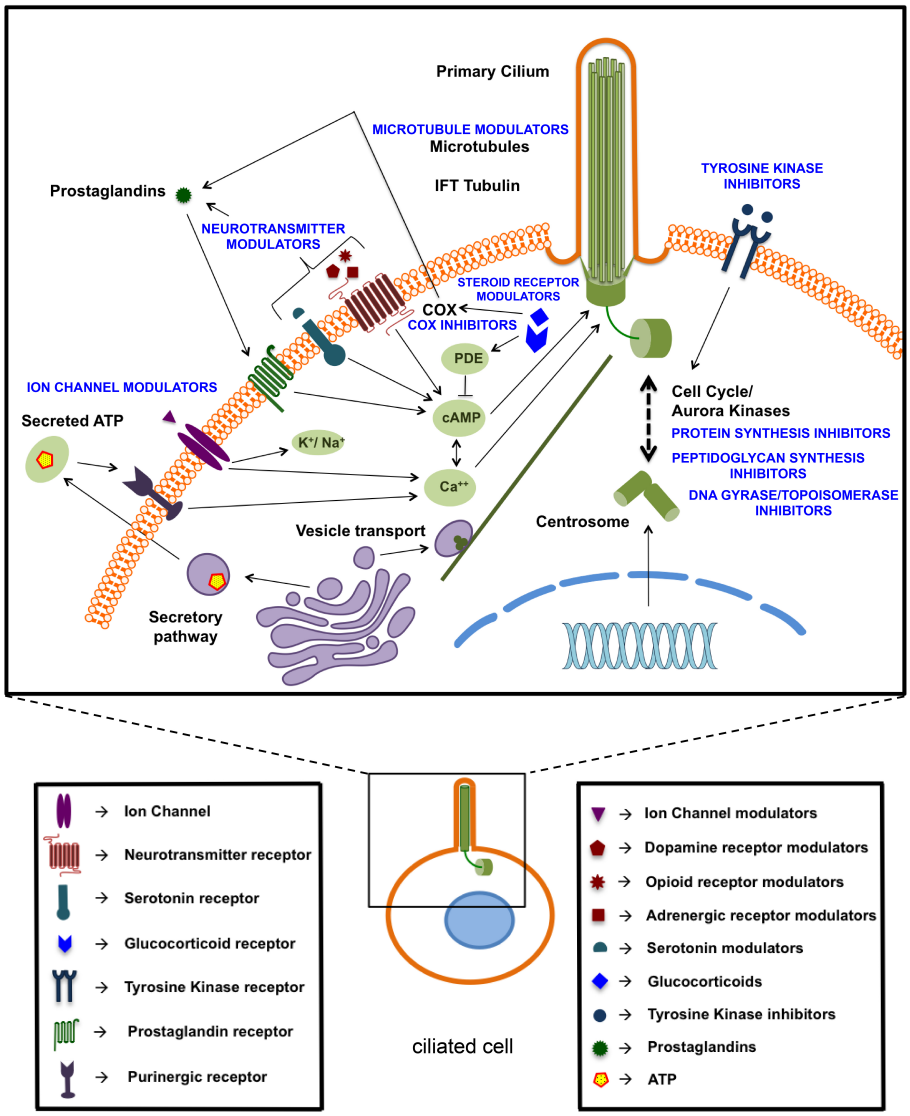

Figure 2.8. Schematic overview of identified ciliogenic compounds based on their potential targets or putative mechanism of action.

ciliogenesis [13], suggesting that the ciliogenic effect of some of these compounds may be indirect and may be downstream of cell cycle arrest. Nevertheless, our findings that deciliation of compound-treated cells restores cell proliferation strongly suggest that the compound-induced ciliogenesis plays an active role in the antiproliferative effects of these compounds.

Our findings that many common drugs have the ability to restore the primary cilium in cancer cell line models may provide interesting new insights in the spectrum of actions of these compounds and may warrant further investigation into application of some of these compounds in future antineoplastic approaches. They also promote the concept of harnessing the therapeutic potential of existing drugs for a novel use, generally referred

to as drug repurposing [64-67]. Development of a new drug is a complex, time-consuming and costly process, mainly due to pharmacological hurdles like bioavailability, solubility, stability, toxicity etc., which form bottlenecks in the therapeutic development process. This can be overcome by drug repurposing, a strategy that reduces the time frame, decreases the costs and improves the success rate by redirecting existing drugs for a new indication. Most of the drugs from the Pharmakon 1600 Library have already been tested for safety in humans and data is available on their pharmacology, formulation and toxicity, paving the way for an accelerated development of cilia-based therapeutics. The success of our screening approach also sets the stage to screen other libraries of new compounds and to establish these as potential novel antineoplastic agents and/or agents exploitable in other cilium-related diseases, including classical ciliopathies.

2.5. Materials and methods

2.5.1. Cell lines

All cell lines were obtained from ATCC. A549 (lung cancer), UMRC2 (kidney cancer) and SUM159 (breast cancer) were maintained in DMEM medium (Life Technologies) supplemented with 10% FBS (Life Technologies). CFPAC-1 (pancreatic cancer) cells were cultured in RPMI-1640 medium (Life Technologies) supplemented with 10% FBS. L3.6 (pancreatic cancer) cells were grown in RPMI-1640 medium supplemented with 10% FBS and 2mM L-Glutamine (Life Technologies). All cell lines were incubated in a humidified incubator at a temperature of 37°C and 5% levels of CO_2.

2.5.2. Compound Library screening

CFPAC-1 cells were seeded in 96-well microplates at a density of 10,000 cells/well containing 200 µl culture medium supplemented with 10% FBS and incubated for 48 hours in a humidified incubator (37°C and 5% CO2), after which medium was refreshed with 200 µl of RPMI-1640 medium containing 2% FBS. The Pharmakon 1600 chemical library consisting of 1600 clinically evaluated compounds and marketed drugs was procured from MicroSource Discovery Systems Inc. (U.S.A). The compounds were dissolved as 10 mM stock solutions in DMSO and added to culture medium to a final concentration of 10 µM. Media and compounds were refreshed on the fourth day after the initial addition of the compounds. After 8 days of compound incubation, the cells were chemically fixed and stained with anti-acetylated tubulin antibody (Sigma, Cat No.

T6793-.5ML), which stains the ciliary axoneme. A fluorescent secondary antibody (Life Technologies, AlexaFluor 488, Cat No. A21145) was used against the primary antibody. The nuclei were counterstained with Hoechst-33258 (Cat No: 382061, Calbiochem). 20 randomized fields per well were imaged at a single plane of focus at 20X magnification in DPBS (Sigma) to reduce auto-fluorescence from the medium and to minimize signal-to-noise ratio. Images acquired by the IN Cell Analyzer 2000 (GE Healthcare) were analyzed using IN Cell Developer software (GE Healthcare).

2.5.3. Confocal microscopy

CFPAC-1 cells were seeded on glass coverslips in 12-well plates containing 1 ml of culture medium per well. At 30% confluency, cell media was replaced by low serum medium containing 2% FBS. After 8 days of compound treatment, cells were fixed with 4% Formaldehyde (Merck), permeabilized with 0.1% Triton X100 (Merck) in DPBS, blocked with 1% BSA (Applichem) in DPBS, and incubated with 1:1000 dilution of anti-acetylated tubulin antibody or 1:500 dilution of anti-IFT88 antibody (Cat. No. 13967-1-AP, Proteintech) for 1h, followed by incubation with 1:1000 dilution of fluorescent secondary antibody for 1h. Nuclei were counterstained with DAPI (Vector Laboratories, Vectashield (Cat. No. H-1500). Images of primary cilia were captured by acquiring Z-stacks using either a Bio-Rad Radiance or Nikon C2 Eclipse Ti-E confocal laser scanning microscope by 40X or 60X oil immersion lenses. All drugs selected for confocal reconfirmation experiments were purchased from Sigma, except for Almotriptan Malate and Sirolimus which were obtained from Selleckchem. Gefitinib was from Invivogen, Cefprozil Monohydrate from Abcam and Imexon from MicroSource Discovery Systems Inc.

2.5.4. Confirmation of ciliogenesis in other cancer cell lines

A549, UMRC2, SUM159 and L3.6 cells were treated as indicated and images were acquired as previous. Image stacks captured by confocal microscope were processed and analyzed for cilia percentage and cilium length using ImageJ software.

2.5.5. Chemical deciliation

CFPAC-1 cells treated with ciliogenic compounds were exposed to 4 mM chloral hydrate (Cat. No. C8383-100G, Sigma) for 24h to remove cilia [68]. Following deciliation, chloral

hydrate was washed out and the cells were allowed to grow in fresh culture medium for 24h before BrdU addition and measurement of proliferation.

2.5.6. Proliferation assays

Spheroid assay - Tumor spheroids were formed on agarose-coated (1%) 96-well plates by seeding L3.6 pancreatic cancer cells in 200 µl of culture medium. The plates were kept undisturbed in a humidified incubator at 37°C and 5% CO_2 for 4 days to facilitate spheroid formation. Treatment with compounds was started when the cells aggregated to form spheroids. Compounds and 50% of the medium were refreshed every 4th day. Images were captured at the beginning of treatment with 5X objective mounted on an inverted light microscope. Imaging was done every 4 days for 20 days from the start of treatment. The images were analyzed by ImageJ software (rsb.info.nih.gov/ij) to calculate the differences in the volume of spheroids at different time points.

BrdU incorporation assay - BrdU incorporation assay was performed using 5-Bromo-2-deoxy-uridine Labeling and Detection Kit III (Cat. No. 11444611001, Roche) according to manufacturer's instructions.

2.5.7. Cell Cycle Analysis

CFPAC-1 cells were plated at a density of 300,000 cells/well of a 6-well plate and allowed to grow for two days. This was followed by compound treatment for 48h and harvesting of cells by trypsinization. Cells were fixed with 4% formaldehyde and permeabilized in 0.1% Triton X100 solution. The permeabilized cells were washed with PBS and stained with Vybrant DyeCycle Green Stain (Cat. No. V35004, ThermoFisher Scientific). Cell cycle data was acquired by measuring at least 10,000 events per sample with BD FACSCanto Flow Cytometer (Becton Dickinson). Data was analyzed using FlowJo software (Becton Dickinson) and the cell cycle distribution was calculated by ImageJ software.

2.5.8. Statistical Analysis

GraphPad Prism version 6 for Mac OS X (GraphPad Software, San Diego, California, USA) was used for statistical analysis. All data are expressed as mean ±SEM. Differences between two groups were assessed using the t-test. To determine differences between more than two groups, two-way ANOVA was used followed by Dunnett's multiple

comparisons test. Differences with p values of < 0.05 were considered to be statistically significant.

2.6. Acknowledgements

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CHAPTER 3

Drug-induced ciliogenesis in pancreatic cancer cells is facilitated by the secreted ATP-purinergic receptor signaling pathway

Drug-induced ciliogenesis in pancreatic cancer cells is facilitated by the secreted ATP-purinergic receptor signaling pathway

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3.1. Abstract

Malignant transformation of cells is often accompanied by the loss of the primary cilium, a protruding microtubule-based sensory organelle, suggesting that it plays an "onco-suppressive" role. Therefore, restoration of the primary cilium is being explored as a new therapeutic approach to attenuate tumor growth. Recently, several commonly used chemotherapeutic drugs have been identified to induce the primary cilium in pancreatic cancer cells. The mechanisms by which these drugs re-express the cilium remain, however, enigmatic. Here, evaluation of a panel of diverse ciliogenic drugs on pancreatic cancer cell models revealed a significant positive relationship between drug-induced extracellular ATP, released through pannexin channels, and the extent of primary cilium induction. Moreover, cilium induction by these drugs was hampered in the presence of the ATP degrading enzyme, apyrase, and in the presence of the pan-purinergic receptor inhibitor, suramin. Our findings reveal that ciliogenic drug-induced re-expression of the primary cilium in pancreatic cancer cells is, at least in certain contexts, dependent on a hitherto unrecognized autocrine/paracrine loop involving the extracellular ATP-purinergic receptor signaling pathway that can be exploited in a therapeutic approach targeting at restoring the primary cilium.

3.2. Introduction

Primary cilia are microtubule-based, antenna-like structures present on the surface of a wide variety of mammalian cells [1]. They serve as platforms for key signaling pathways

that are critical for development and tissue homeostasis by specifically expressing crucial entities like ion channels, transporter proteins and receptors [2-4]. Hence, defects that compromise ciliary structure and/or function can have profound consequences for a cell [4]. Cilia dysfunction is considered to be a common event in many cancer types, including melanoma, breast cancer and pancreatic cancer [4]; and is associated with poor prognosis in patients [4-8]. Recently, a high-throughput screening based on the pancreatic CFPAC-1 cancer cell line delineated chemotherapeutic compounds with the ability to induce primary cilia in cancer cells, and paved the way to explore cilium induction as a hitherto unexploited concept in the context of cancer chemotherapy [9].

Armed with such novel ciliogenic drugs, we aimed to explore the mechanisms underlying cilium-induction in pancreatic cancer cells. Recent discoveries demonstrate that several anticancer modalities, including chemotherapeutic drugs, cause secretion of ATP from cancer cells [10-14]. Initially delineated in the context of neurotransmission, extracellular ATP now has emerged as an important messenger in a number of signaling paradigms [15, 16]. It facilitates cell-to-cell communication in both autocrine and paracrine manners in various pathological conditions [10, 15]. In the context of cancer, extracellular ATP has recently emerged as an important damage-associated molecular pattern (DAMP) [17], modulating both chemo-attraction and activation of innate immune cells in a paracrine manner [12, 18, 19]. On the other hand, autocrine signaling elicited by extracellular ATP can also facilitate pro-cancerous processes e.g. increased proliferation and migration/invasion. Thus, depending on the signaling context and target cells involved, extracellular ATP can exert both pro- and anti-cancerous effects [15, 16, 20]. Two mutually-exclusive pathways have been demonstrated to facilitate trafficking of extracellular ATP after treatment with anticancer therapy [17] i.e. the classical secretory pathway (in case of photodynamic therapy) and the pannexin channels-based secretion (in case of chemotherapy and/or targeted therapy) [18, 21]. After release or secretion, extracellular ATP tends to signal through the P2 purinergic receptors on the surface of (target) cells [15].

Interestingly, two decades ago some studies had reported that exogenous addition of extracellular ATP can improve ciliary beat frequency (CBF) in epithelial cells derived from frog palate and esophagus [22, 23]. The modulation of CBF was further shown to

occur through activation of purinergic receptors present on the cilium [3, 24, 25]. Consistent with these findings, mutant mice lacking purinergic receptors showed a significant reduction in CBF [26]. Collectively, these findings hinted towards a bi-directional link between primary cilia and extracellular ATP or purinergic receptor signaling, that had not been probed in the context of cancer cells so far.

Following the above cues, we aimed to investigate to what extent and how cilia-inducing chemotherapeutic drugs (hereafter referred to as ciliogenic drugs) promote ciliogenesis in pancreatic cancer cells through the extracellular ATP-purinergic receptor signaling pathway. Here we show that in CFPAC-1 pancreatic ductal cancer cells that were used for the original screening for ciliogenic chemotherapeutics, several ciliogenic compounds exert their cilium-promoting effects at least in part through the extracellular ATP-purinergic receptor signaling pathway.

3.3. Results

3.3.1. Ciliogenic chemotherapeutic drugs induce secretion of ATP in pancreatic cancer cells

First of all, we wanted to assess whether extracellular ATP levels in fact correlate with re-expression of the primary cilium in cancer cells by ciliogenic chemotherapeutic drugs. To this end, we first investigated to what extent these drugs cause release/secretion of ATP in the extracellular medium. To address this, human pancreatic cancer cells (CFPAC-1) were treated with a panel of 22 drugs that were previously shown to induce ciliogenesis in these cells [9]. The drug esomeprazole, which is incapable of inducing cilia [9], was included as negative control. Interestingly, 6 out of 22 drugs significantly increased the levels of extracellular ATP as compared to untreated control cells (Fig 3.1A). These results were reconfirmed in another pancreatic cancer cell line model PANC-1 for a selection of compounds (Fig 3.S1). Although most other ciliogenic compounds also induced a certain degree of ATP secretion/release, it was not statistically significant. Interestingly, a correlation analysis showed that drug-induced re-expression of primary cilia positively correlates (significantly) with increased secretion/release of ATP in these cancer cells (Fig 3.1B). These observations confirmed that certain potent ciliogenic drugs also tend to cause increased release/secretion of ATP.

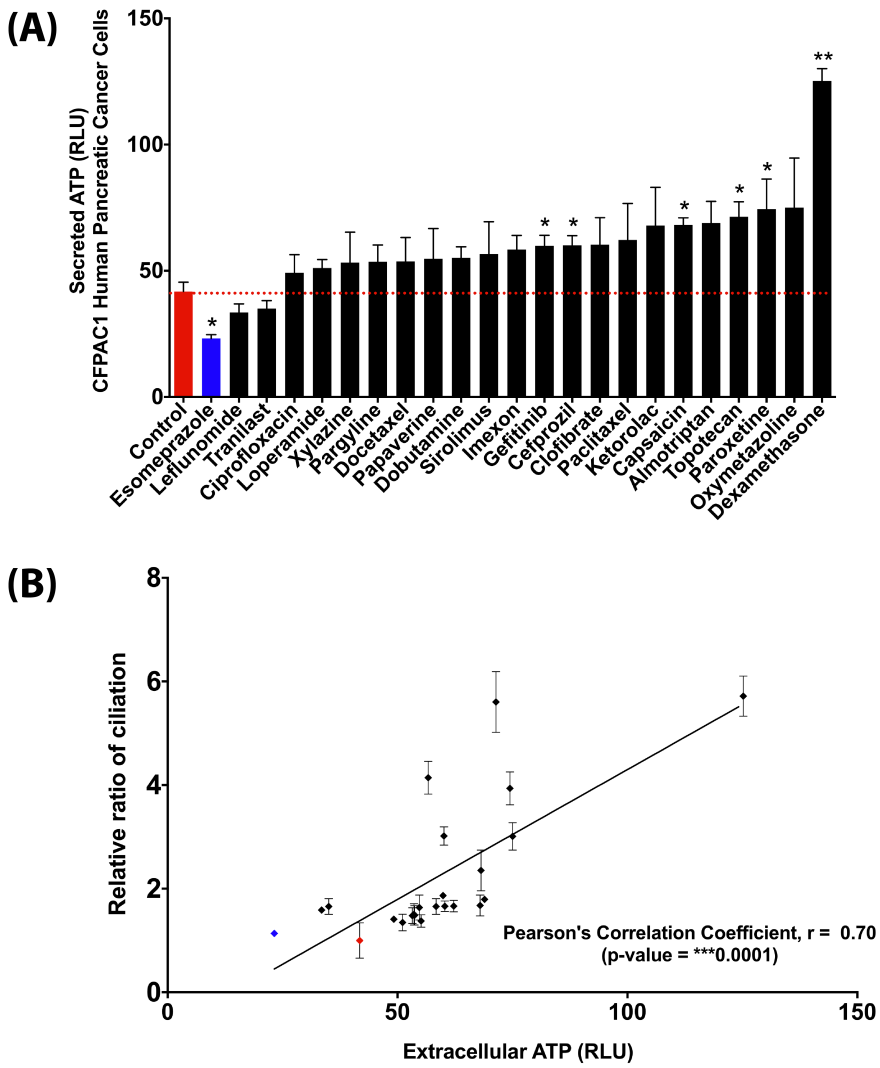

Figure 3.1. Ciliogenic chemotherapeutics tend to induce extracellular ATP in cancer cells. Quantitative and correlative analysis of extracellular ATP upon exposure of CFPAC-1 cells to a panel of ciliogenic drugs (black bars and dots) and a non-ciliogenic drug (blue bar and dot) at 2 micromolar concentration for 96 hours. (A) The drugs are ranked in ascending order of their potency to release extracellular ATP into the culture medium as assessed by the measurement of bioluminescence based on luciferin-luciferase conversion principle. The red dotted line represents the basal level of extracellular ATP in the medium (represented as relative luminescence units or RLUs). (B) Regression analysis between extracellular ATP and cilia induction. Correlation was calculated by using Pearson correlation coefficient analysis. Data are presented as mean ± SEM, *p ≤ 0.05, **p ≤ 0.005, ***p ≤ 0.0005.

3.3.2. Exogenous ATP induces primary cilia in pancreatic cancer cells

The above observations spurred us to assess whether exogenous ATP can in fact modulate ciliogenesis. Therefore, we exposed untreated CFPAC-1 cells to increasing concentrations of exogenously added ATP and visualized the primary cilium by confocal microscopy. A significant increase in the percentage of ciliated cells was observed already at nanomolar concentrations of exogenously added ATP. At higher micromolar concentrations the increase was less pronounced but still significant (Fig 3.2A and 3.2B). A similar effect was seen in PANC-1 cells (Fig 3.S2A and 3.S2B). These results show that

exogenous ATP enhances ciliogenesis in pancreatic cancer cells already at low concentrations that are in the range of the concentrations measured in the cultures after drug treatment (10 - 125 nM), suggesting a causative link between secreted ATP and cilia induction in pancreatic cancer cells.

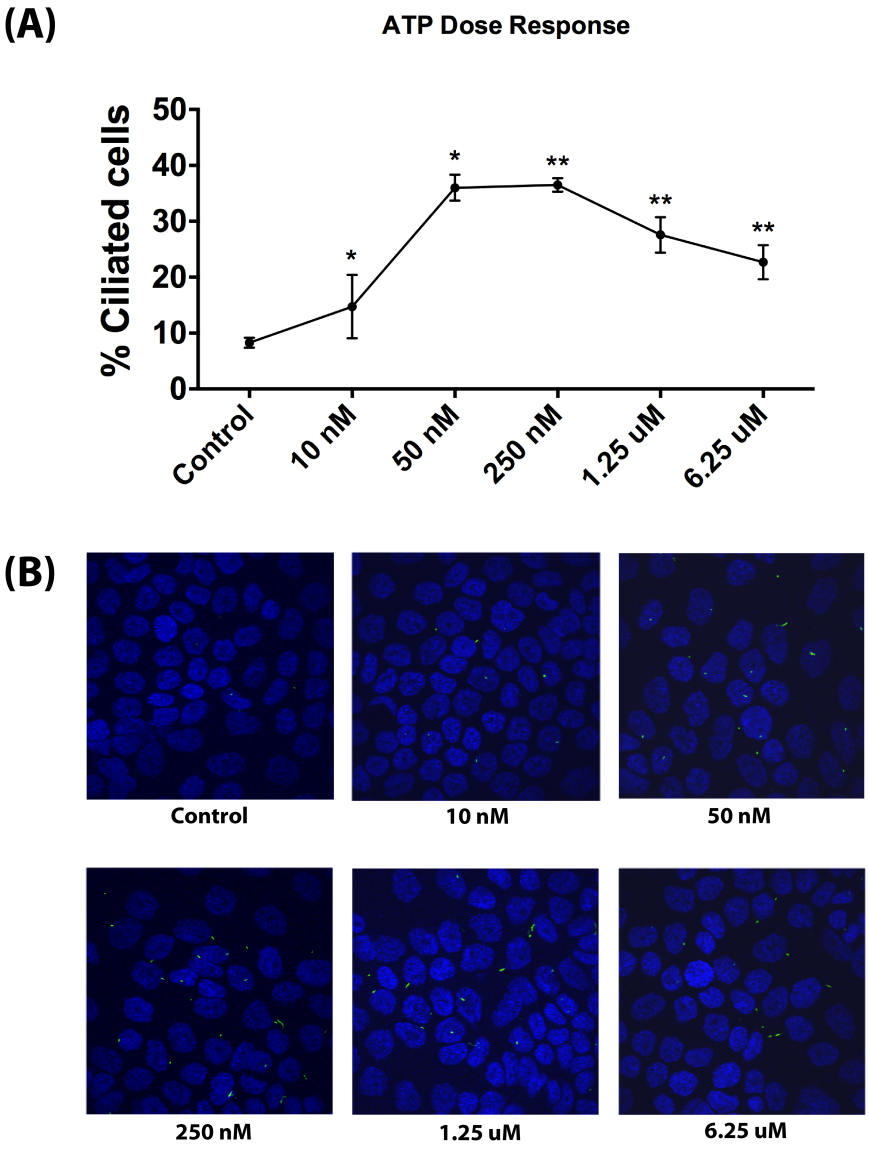

Figure 3.2. Effect of exogenous ATP on cilia induction in CFPAC-1 cells. (A) Quantitative analysis of ciliogenesis upon treatment of cells with exogenous ATP at increasing concentrations, as assessed by confocal fluorescence microscopy. (B) Representative images of cells showing the effect of exogenous ATP on ciliation. Nuclei were stained with DAPI (blue) and cilia with an antibody against the cilium marker acetylated tubulin (green). All images were captured using Olympus Fluoview confocal microscope using a 40X objective lens. Data are presented as mean ± SEM, *p ≤ 0.05, **p ≤

3.3.3. Degradation of drug-induced extracellular ATP suppresses ciliogenesis in pancreatic cancer cells

To corroborate the link between secreted ATP and cilium induction, we assessed the ability of two potent ciliogenic and ATP-releasing compounds (dexamethasone and gefitinib) to induce ciliogenesis in the presence of apyrase, a known ATP degrading enzyme. The non-ciliogenic compound Esomeprazole, which also displayed a lack of ability to induce ATP secretion, was used as a negative control. To this end, we applied an immunofluorescence microscopy-based phenotypic imaging strategy in a 96-well

format using an IN Cell Analyzer, conceived by us previously [9]. In the presence of apyrase, the ability of both ciliogenic drugs (dexamethasone and gefitinib) to increase the percentage of ciliated CFPAC-1 and PANC-1 cells was blunted, as compared to cancer cells treated in the absence of this ATP degrading enzyme (Fig 3.3A and 3.S3A). These data were further substantiated by confocal microscopy (Fig 3.4B and 3.S3B). These results provide further evidence that extracellular ATP is involved in cilium induction and thereby point towards the involvement of a secreted ATP-dependent autocrine mechanism in the re-expression of primary cilia in pancreatic cancer cells, by a subset of ciliogenic drugs.

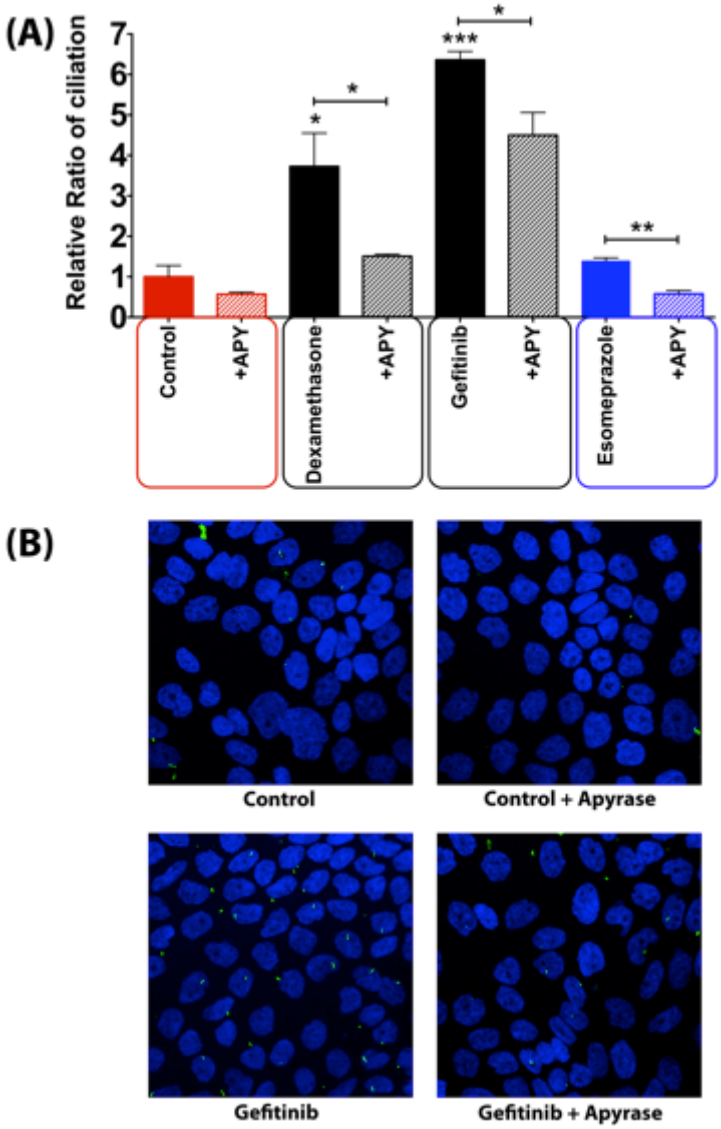

Figure 3.3. Effect of apyrase-mediated extracellular ATP degradation on ciliogenesis in CFPAC-1 cells exposed to ciliogenic drugs.
(A) Quantification of the effect of apyrase treatment on ciliogenesis.
(B) Representative images showing the effect of apyrase on ciliogenesis of cells exposed to the indicated drugs. Nuclei were stained with DAPI (blue) and cilia with an antibody against the cilium marker acetylated tubulin (green). All images were captured using Nikon C2 Eclipse Ni-E confocal microscope using a 60X objective lens. Data are presented as mean ± SEM, *p ≤ 0.05, **p ≤ 0.005, ***p ≤ 0.0005.

3.3.4. Pannexin channels mediate the secretion of extracellular ATP by ciliogenic drugs

Beyond the above discussed effects which confirm that drug-induced ATP release affects ciliogenesis, chemotherapeutic drugs tend to also modulate the proliferation and

survival of cancer cells – processes that have ability to modulate/exploit extracellular ATP. To this end, we deemed it crucial to analyze the overall correlation between secreted/released ATP and drug-induced effects on proliferation/survival. We observed an overall reduction in cellular proliferation following ciliogenic drug treatment, which interestingly correlated with an increase in the secretion/release of ATP (Fig 3.4A) thereby exposing a negative correlation between proliferation and extracellular ATP in this system. On the other hand, although most of the ciliogenic drugs compromised cellular survival to a certain extent, no statistically significant correlation was observed between cellular survival and ATP secretion (Fig 3.4B), suggesting the possibility of the existence of an active ATP secretion pathway.

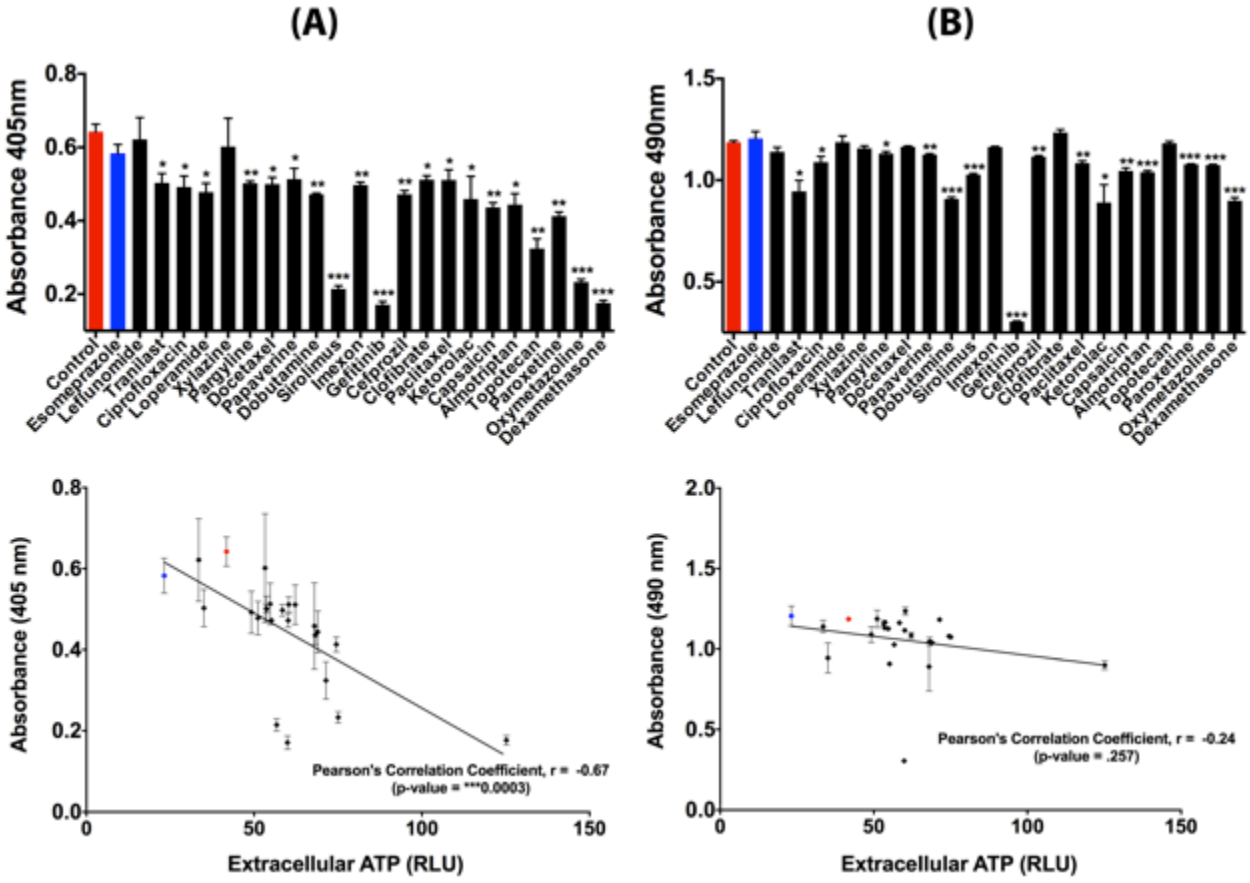

Figure 3.4. Effect of secreted ATP on proliferation and survival of CFPAC-1 pancreatic cancer cells. (A) Quantitative analysis of cell proliferation upon secretion of ATP by exposure of cells to ciliogenic chemotherapeutic drugs (black bars and dots) and a non-ciliogenic drug (blue bar and dot). Changes in proliferation were measured by a BrdU incorporation assay. Lower panel shows correlative analysis between cell proliferation and secreted ATP. (B) Quantitative analysis of cell viability upon exposure of cells to ciliogenic chemotherapeutic drugs and a non-ciliogenic drug as measured by MTS assay. Lower panel displays the correlation between cell survival and secreted ATP in treated cells. Correlation was calculated by using Pearson correlation coefficient analysis. Data are presented as mean ± SEM, *p ≤ 0.05, **p ≤ 0.005, ***p ≤ 0.0005.

Although the cellular survival assay suggested that at least some extracellular ATP in our set-up might be passively released, the lack of a statistically significant correlation between survival and ATP release (Fig 3.4B) prompted us to explore whether ATP is secreted by the studied pancreatic cancer cells in an active manner; and to what extent this is crucial for ciliogenesis. To investigate the involvement of the two main ATP secretion mechanisms [17] we treated cells with well-established small molecule inhibitors each targeting different players in putative ATP secretion pathway

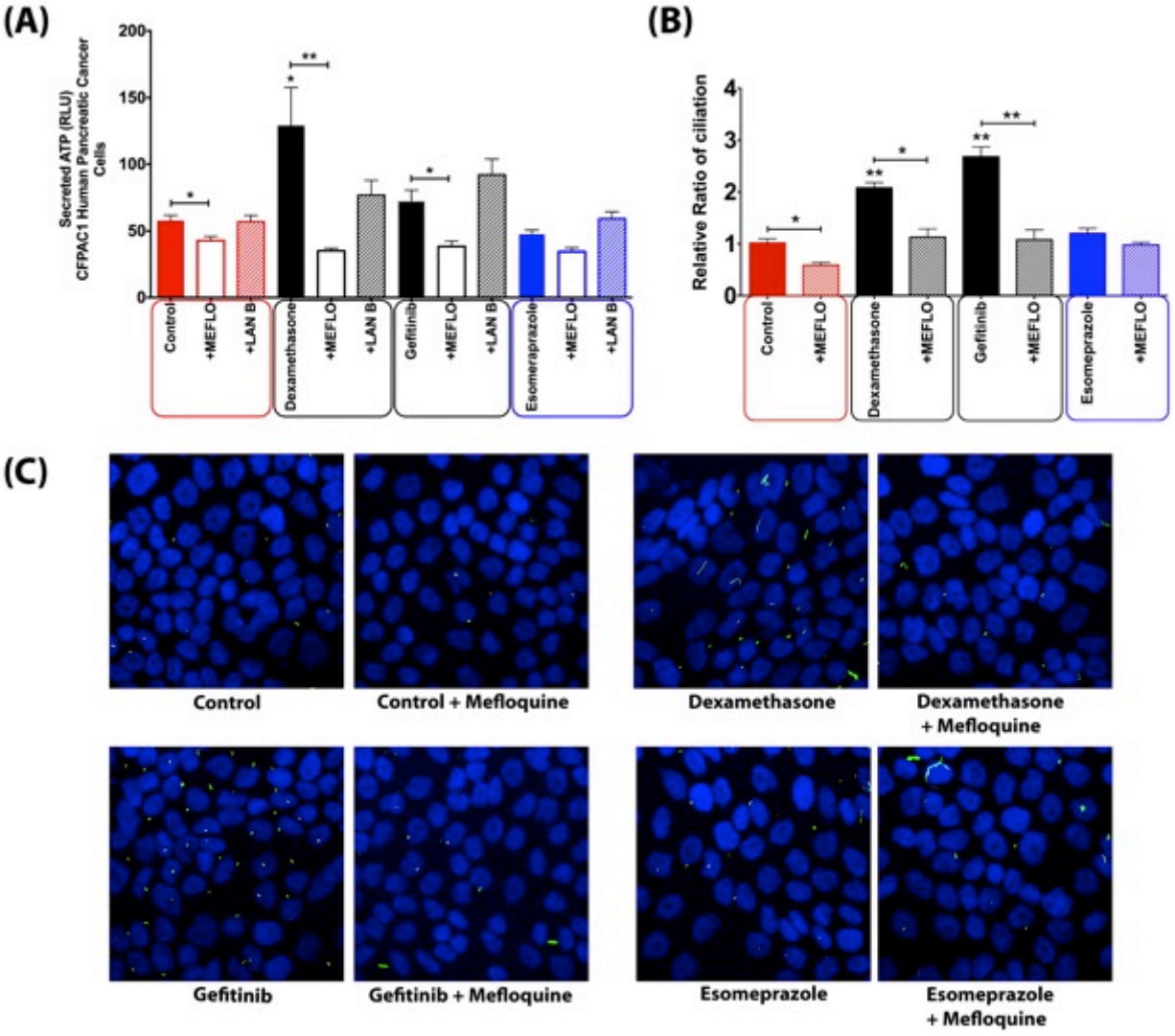

Figure 3.5. Effect of inhibition of different intracellular trafficking pathways on the concentration of extracellular ATP in CFPAC-1 cells treated with ciliogenic drugs. (A) Quantification of extracellular ATP in cells treated with pannexin channel blocker (mefloquine), vesicular transport inhibitor (latrunculin B), inhibitor of microtubule-dependent transport (nocodazole). (B) Quantification of the effect of mefloquine on ciliogenesis in CFPAC1 cells. (C) Representative images showing the effect of pannexin channel blockade on ciliogenesis in CFPAC-1 cells exposed to a selection of ciliogenic drugs. Nuclei were stained with DAPI (blue) and cilia with an antibody against the cilium marker acetylated tubulin (green). All images were captured using Nikon C2 Eclipse Ni-E confocal microscope using a 60X objective lens. Data are presented as mean ± SEM, *p ≤ 0.05, **p ≤ 0.005, ***p ≤ 0.0005.

Vesicular transport mediated by actin was inhibited by the actin polymerization inhibitor latrunculin B; and pannexin-based release was inhibited by the pannexin channel inhibitor mefloquine. Latrunculin B pre-treatment failed to reduce drug-induced ATP secretion (Fig 3.5A), thereby ruling out a major role of vesicular and/or secretory anterograde transport in ATP secretion in this set-up. However, when pannexin channels were blocked with mefloquine, a significant reduction in the extracellular ATP was observed, indicating the involvement of this mechanism in ATP secretion induced by certain drugs (Fig 3.5A). The role of pannexins-based ATP secretion in our set-up was further corrobrated by the observation that CFPAC-1 cells pre-treated with mefloquine and subsequently exposed to ciliogenic drugs showed a significant reduction in the percentage of ciliated cells (Fig 3.5B and 3.5C).

3.3.5. P2 purinergic receptors are involved in drug-induced ciliogenesis

As mentioned previously, extracellular ATP tends to largely signal through the P2 purinergic receptors. Since the increased extracellular ATP had a positive effect on ciliogenesis, we decided to probe the involvement of the extracellular ATP-P2 purinergic receptor link in the induction of primary cilia in CFPAC-1 cells by ciliogenic

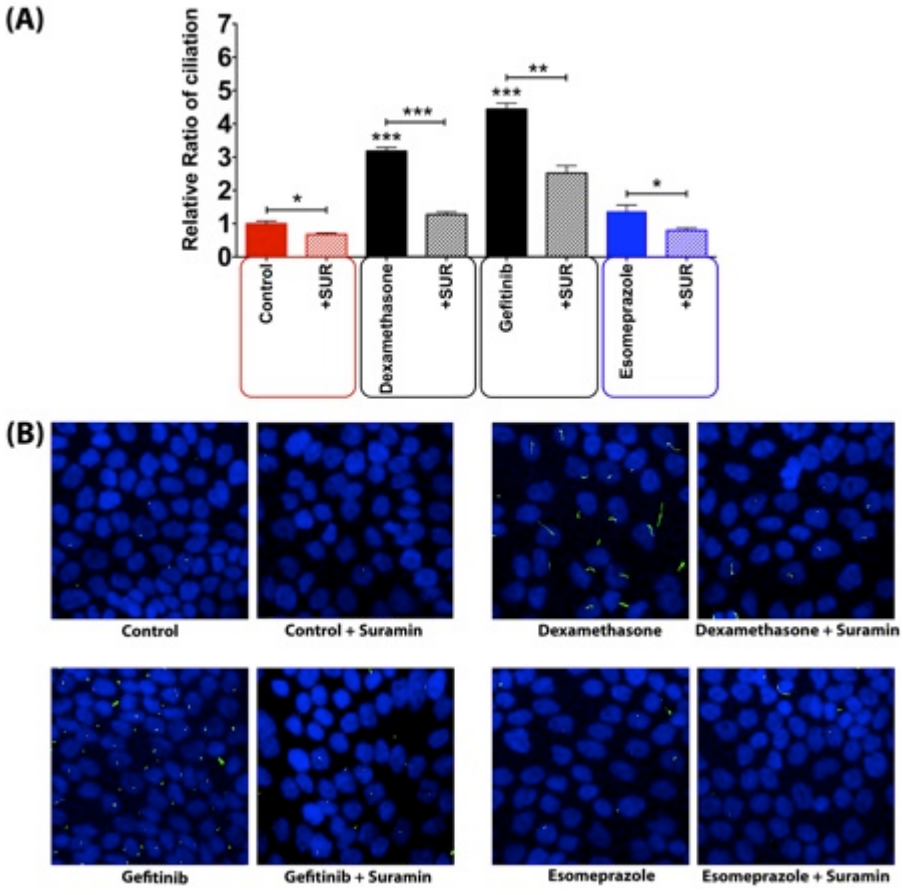

Figure 3.6.
P2 purinergic receptor blockade reduces ciliogenesis.
(A) Effect of suramin, a blocker of P2 purinergic receptors on ciliogenesis in CFPAC-1 cells exposed to ciliogenic drugs.
(B) Representative images of CFPAC-1 cells showing the effect of suramin on ciliation in untreated and treated cells. Nuclei were stained with DAPI (blue) and cilia with an antibody against the cilium marker acetylated tubulin (green). All images were captured using Nikon C2 Eclipse Ni-E confocal microscope using a 60X objective lens. Data are presented as mean ± SEM, *p ≤ 0.05, **p ≤ 0.005, ***p ≤ 0.0005.

drugs. To this end, we exposed cells treated with representative ciliogenic drugs to suramin, a pan-P2 receptor inhibitor, and examined to what extent this interferes with ciliogenesis. Indeed, a significant suppression of ciliogenesis was observed in cells treated with the drugs when suramin was present (Fig 3.6A and 3.6B). Thus, our data show that both degrading ATP (thus removing the ligand) or inhibiting its main receptor exert the same effect, suggesting that the extracellular ATP-P2 purinergic receptor signaling arc is involved in the induction of primary cilia in pancreatic cancer cells by a subset of ciliogenic drugs.

3.4. Discussion

In a recent study we had shown that various commonly used chemotherapeutic drugs restore ciliogenesis in cancer cells [9]. Since the cilium uses the same structural elements as the centrosome that is required for cell division and proliferation, ciliogenesis is believed to put a brake on the proliferation of cells, including many cancer cells. This is also the reason behind cilium induction being regarded as a novel therapeutic mechanism [9]. Here, we demonstrate that certain drugs promote ciliogenesis, at least in part, through a hitherto unrecognized autocrine/paracrine loop involving the extracellular ATP-purinergic receptor signaling pathway. We found that a subset of ciliogenic compounds stimulate ATP release, which positively correlates with their ciliogenic ability. Consistent with previous studies [18, 21], using chemical inhibitors targeting different potential players in ATP-secretion pathways we found that specific ciliogenic drugs stimulate the active secretion of ATP mainly through pannexin channels. This active ATP secretion pathway might co-exist with some passive release of ATP, at least in the case of certain ciliogenic drugs that induce cell death in a fraction of the cancer cells. Extracellular ATP in turn promotes ciliogenesis as revealed by the treatment of cells with varying concentrations of exogenous ATP, by blocking P2 purinergic receptors, which are known to mediate many effects of extracellular ATP [15] and by co-treatment with the ATP-degrading enzyme apyrase. Thus, a picture emerges in which the extracellular ATP may act in both an autocrine manner (ATP secreted by a live cell that acts on the same cell) and a paracrine manner (ATP released from dead/dying cells or secreted by live cells that acts on other live cells) to induce ciliogenesis in pancreatic cancer cells. Figure 3.7 illustrates the involvement of this autocrine/paracrine loop of extracellular ATP in ciliogenesis.

This involvement of the extracellular ATP-purinergic receptor pathway in the cilium-inducing effect of certain chemotherapeutic drugs is of particular interest in view of the emerging central role of purinergic signaling in many cellular events including inflammation and wound healing [28-30]. Our observation of a statistically significant negative correlation between extracellular ATP levels and overall proliferation of pancreatic cancer cells is particularly intriguing in view of the divergent effects of extracellular ATP on cell proliferation [15, 31-33] and suggest that the mitigating effects of extracellular ATP-induced ciliogenesis on proliferation may at least partially account for this discrepancy. This notion needs proper molecular analysis and warrants further examination in a tumor context in vivo, to what extent this newly delineated function of extracellular ATP-purinergic signaling contributes to the anticancer effects of the studied drugs in pancreatic and other cancers.

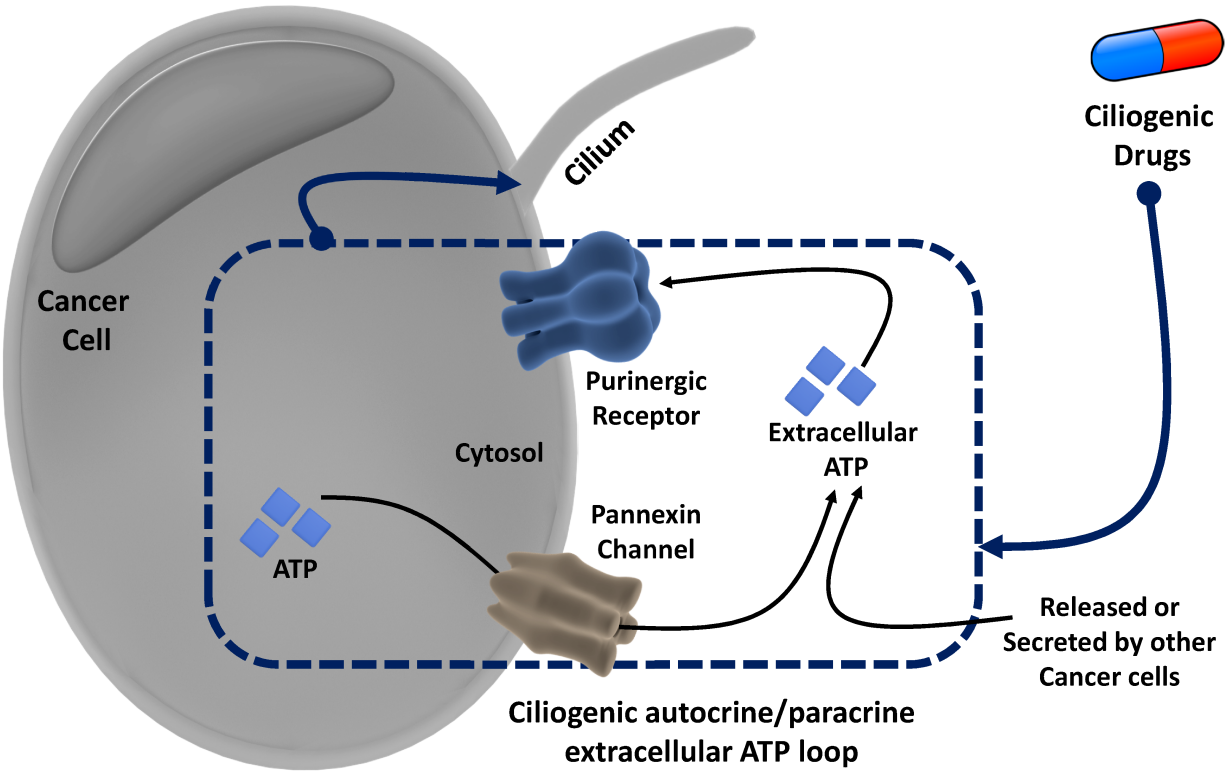

Figure 3.7. Overview of the proposed autocrine/paracrine loop model of extracellular ATP-mediated ciliogenesis by ciliogenic chemotherapeutics through activation of the pannexin pathway and purinergic signaling.

While we have provided evidence for the role of autocrine/paracrine signaling by the extracellular ATP in drug-induced ciliogenesis; it should be considered that this pathway may not be the only existing mechanism. The action of chemotherapeutic drugs tends to be pleiotropic, consisting of both on-target as well as off-target therapeutic effects. In view of the complexity of cilia-inducing signaling it is likely that other signaling

pathways also play a major role. Moreover, considering that extracellular ATP also has a crucial role in the modulation of inflammation [17, 34-37], and as a large number of anti-inflammatory drugs have been reported to induce primary cilia in cancer cells [9], it would be interesting to investigate the relationship between ciliogenesis and inflammation and the role of extracellular ATP in this context. Further elucidation of these and other pathways will be of interest to fully exploit the cilium-inducing property of common chemotherapeutics in a more clinical setting.

3.5. Materials and Methods

3.5.1. Reagents and Drugs

Esomeprazole, Pargyline, Leflunomide, Clofibrate, Ciprofloxacin, Ketorolac, Paroxetine, Dobutamine, Tranilast, Loperamide, Xylazine, Capsaicin, Papaverine, Oxymetazoline, Dexamethasone, Docetaxel, Topotecan, Paclitaxel were purchased from Sigma. Almotriptan Malate and Sirolimus were purchased from Selleckchem, Gefitinib from Invivogen, Cefprozil Monohydrate from Abcam and Imexon from MicroSource Discovery Systems Inc.; ATP, Apyrase, Suramin, Nocodazole and Mefloquine were purchased from Sigma; Latrunculin B was purchased from Abcam.

3.5.2. Cell culture and drug treatments

Both the pancreatic cancer cells CFPAC-1 and PANC-1 were obtained from ATCC. CFPAC-1 cells were cultured in RPMI-1640 medium (Life Technologies) whereas PANC-1 cells were maintained in DMEM medium (Life Technologies). Both media were supplemented with 10% FBS (Life Technologies) and incubated at 37°C in 5% CO_2. For drug treatments, cells were seeded in 96-well plates at a density of 10,000 cells per well containing 200ul of culture medium supplemented with 10% FBS and incubated in a humidified incubator at 37° C and 5% CO_2 for 48 hours. This was followed by refreshment of the medium with culture medium containing 2% FBS. Assays were performed by treating the cells with drugs as indicated: for induction of primary cilia, the cells were treated with ciliogenic drugs. Hydrolysis of ATP was achieved by treating cells with Apyrase. Purinergic receptor signaling in cells was blocked by treatment with Suramin. Disruption of the secretory pathway was carried out by Latrunculin B treatment. Mefloquine was used to block the pannexin pathway.

3.5.3. Immunofluorescence and automated imaging for primary cilia

To stain primary cilia, chemotherapeutics-treated and untreated cells were chemically fixed with 4% Formaldehyde (Merck), permeabilized with 0.1% Triton X100 (Merck) in DPBS, blocked with 1% BSA (Applichem) in DPBS, and incubated with 1:1000 dilution of anti-acetylated tubulin antibody (Sigma) for 1 hour followed by incubation with 1:1000 dilution of a fluorescent secondary antibody (Life Technologies, AlexaFluor 488) for 1 hour. Nuclei were stained with Hoechst-33258 (Calbiochem). Images were acquired using an IN Cell Analyzer (GE Healthcare) at 20X magnification and were analyzed using IN Cell Developer software. In order to minimize the signal-to-noise ratio, 20 randomized fields per well were imaged at a single plane of focus.

3.5.4. Confocal microscopy

CFPAC-1 and PANC-1 cells were seeded on glass coverslips in 12-well plates containing 1 ml culture medium per well and allowed to grow to 30% confluency. This was followed by replacement of culture medium with medium containing 2% FBS and treatment of cells with drugs as previously indicated. To visualize the primary cilium, treated and untreated cells were fixed with 4% formaldehyde, permeabilized with 0.1% Triton X100 in DPBS, blocked with 1% BSA in DPBS, incubated with 1:1000 dilution of anti-acetylated tubulin antibody for 1 hour, followed by incubation with 1:1000 dilution of fluorescent secondary antibody for 1 hour. The coverslips were mounted on glass slides and stained for nuclei with DAPI (Vector Laboratories, Vectashield). Images of primary cilia were captured by acquiring Z-stacks using Olympus FluoView-FV 1000 or Nikon C2 Eclipse Ni-E confocal laser scanning microscope using 60X oil immersion lens.

3.5.5. Measurement of extracellular ATP

Extracellular ATP was measured using an ATP Bioluminescent assay kit (Sigma) based on the luciferin-luciferase conversion principle, according to the manufacturer's instructions, as described previously [27]. Bioluminescence was measured by optical top reading using a FlexStation 3 microplate reader (Molecular Devices Inc., Sunnyvale, CA, USA).

3.5.6. Proliferation assay

Proliferation assay was performed by measuring BrdU incorporation using 5-Bromo-2-deoxy-uridine Labeling and Detection Kit III (Cat No. 11444611001) in accordance with the manufacturer's protocol.

3.5.7. Cell viability assay

The viability of cells in drug treated cultures was determined by measuring the bioreduction of MTS tetrazolium compound using the CellTiter96 AQueous One Solution Cell Proliferation Assay kit (Promega). The assay was performed by following the protocol provided with the kit.

3.5.8. Statistical analysis

Statistical analysis of data was performed using GraphPad Prism version 6 for Mac OS X (GraphPad Software, San Diego, California, USA). All data are expressed as mean ±SEM. Significance level of Student's t-test was set at $p < 0.05$.

3.6. Supplemental data

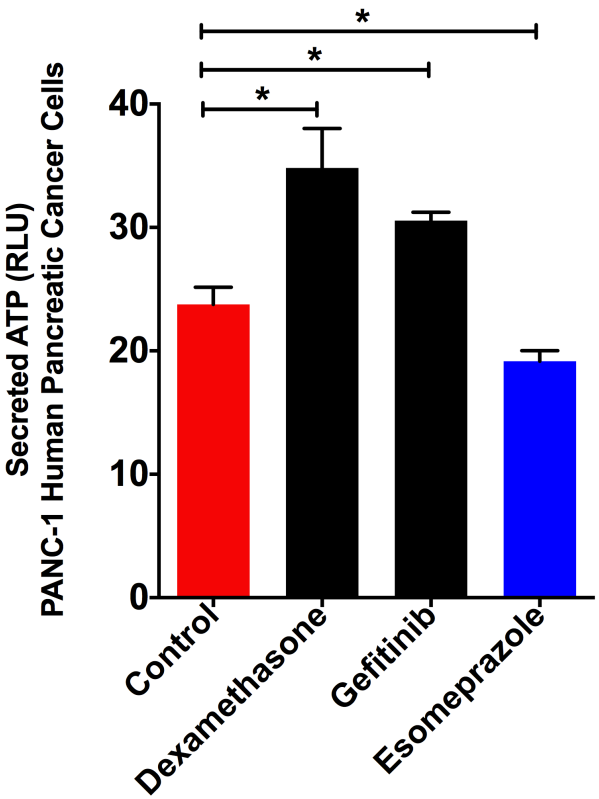

Figure 3.S1. Ciliogenic chemotherapeutics induce extracellular ATP in pancreatic cancer cell line PANC-1. Quantitative analysis of ATP secretion upon exposure of PANC-1 cells to a selection of ciliogenic drugs (black bars) and a non-ciliogenic drug (blue bar) at 2 micromolar concentration for 96 hours (A) as assessed by the measurement of bioluminescence based on luciferin-luciferase conversion principle. Data are presented as mean ± SEM, *p ≤ 0.05, **p ≤ 0.005, ***p ≤ 0.0005.

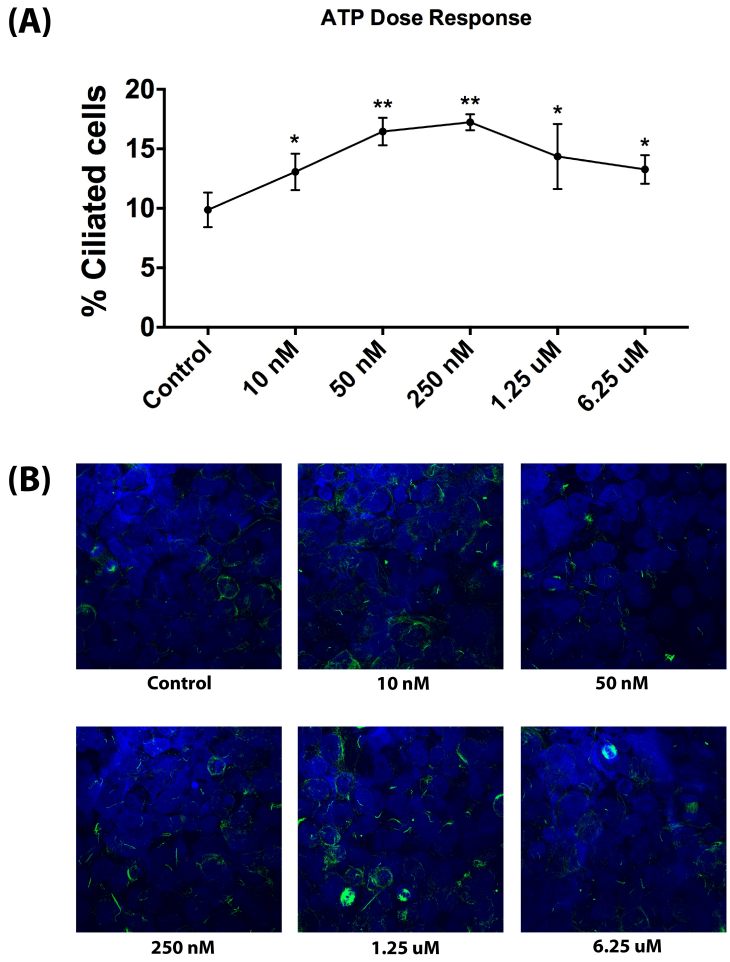

Figure 3.S2. Effect of exogenous ATP on cilia induction in PANC-1 cells. (A) Quantitative analysis of ciliogenesis in cells treated with increasing concentrations of exogenous ATP, as assessed by confocal fluorescence microscopy. (B) Representative images showing the effect of exogenous ATP on ciliation in PANC-1 cells. Nuclei were stained with DAPI (blue) and cilia with an antibody against the cilium marker acetylated tubulin (green). All images were captured using Olympus Fluoview confocal microscope using a 40X objective lens. Data are presented as mean ± SEM, *p ≤ 0.05, **p ≤ 0.005, ***p ≤ 0.0005.

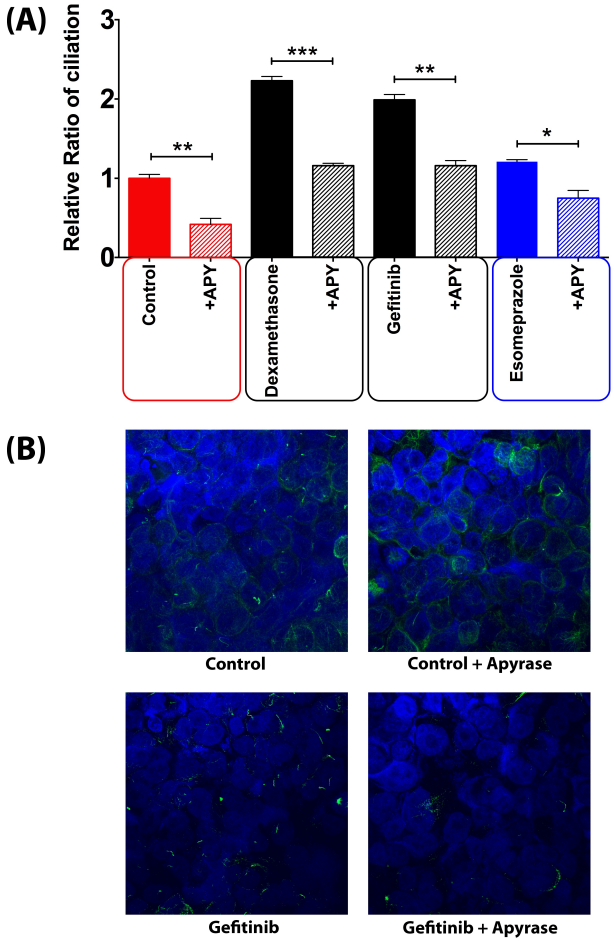

Figure 3.S3. Apyrase-mediated degradation of extracelluar ATP in pancreatic cancer cells PANC-1 exposed to ciliogenic drugs. (A) Quantitative analysis of the effect of apyrase treatment on ciliogenesis. (B) Representative images showing the effect of apyrase on ciliogenesis in cells treated with indicated drugs. Nuclei were stained with DAPI (blue) and cilia with an antibody against the cilium marker acetylated tubulin (green). Data are presented as mean ± SEM, *p ≤ 0.05, **p ≤ 0.005, ***p ≤ 0.0005.

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CHAPTER 4

Identification of kinase inhibitors as modulators of ciliogenesis in cancer cells

Identification of kinase inhibitors as modulators of ciliogenesis in cancer cells

4.1. Introduction

Most mammalian cells have an elongated protruding structure called the primary cilium that is crucial for the ability of the cell to sense signals from the extracellular environment. Accumulating evidence points towards a possible link between these structures and cancer development [1]. However, the precise role of cilia in this context is not yet known. It is unclear whether cilia regress or promote the progression of cancers. A number of reports have documented the loss of cilia in different cancer types that include breast, prostate and pancreatic cancers [2-4]. On the other hand, several studies have provided evidence that presence of cilia is necessary for the survival of certain types of cancers like basal cell carcinoma (BCC), medulloblastoma and a subset of glioblastomas [5, 6]. Cilia might thus act as unique organelles that can play a paradoxical role of either mediating or curbing oncogenesis [7, 8]. Therefore, depending on the cancer type, targeting the cilium by agonists or antagonists to either restore or suppress ciliogenesis can be considered as a novel therapeutic approach to treat cancers. In our previous work, we have developed a cell-based screening approach to identify compounds that have the ability to modulate cilium expression in cells [9]. Here, in view of the reported role of kinases in the regulation of ciliogenesis and their widely applied use in the oncology field, we have used this approach to screen a library of kinase inhibitors to identify cilium modulators. Our results show that kinase inhibitors may either stimulate or repress ciliogenesis in cancer models. Key examples include Herbimycin A and Kenpaullone, which suppress ciliogenesis in cilia-dependent glioblastoma cells and attenuate their cell growth and proliferation, and EGFR/ErbB-2/ErbB-4 Inhibitor (CAS 881001-19-0) and Src Kinase Inhibitor I, which enhance ciliogenesis in poorly ciliated pancreatic cancer cells. These findings provide novel insights in the role of kinases in the regulation of ciliogenesis. They expand our arsenal of tools to modulate ciliogenesis in cancer cells depending on the cilium-dependence of the tumor type, and they advance the concept that modulation of ciliogenesis may be used as a therapeutic strategy to combat different types of cancers.

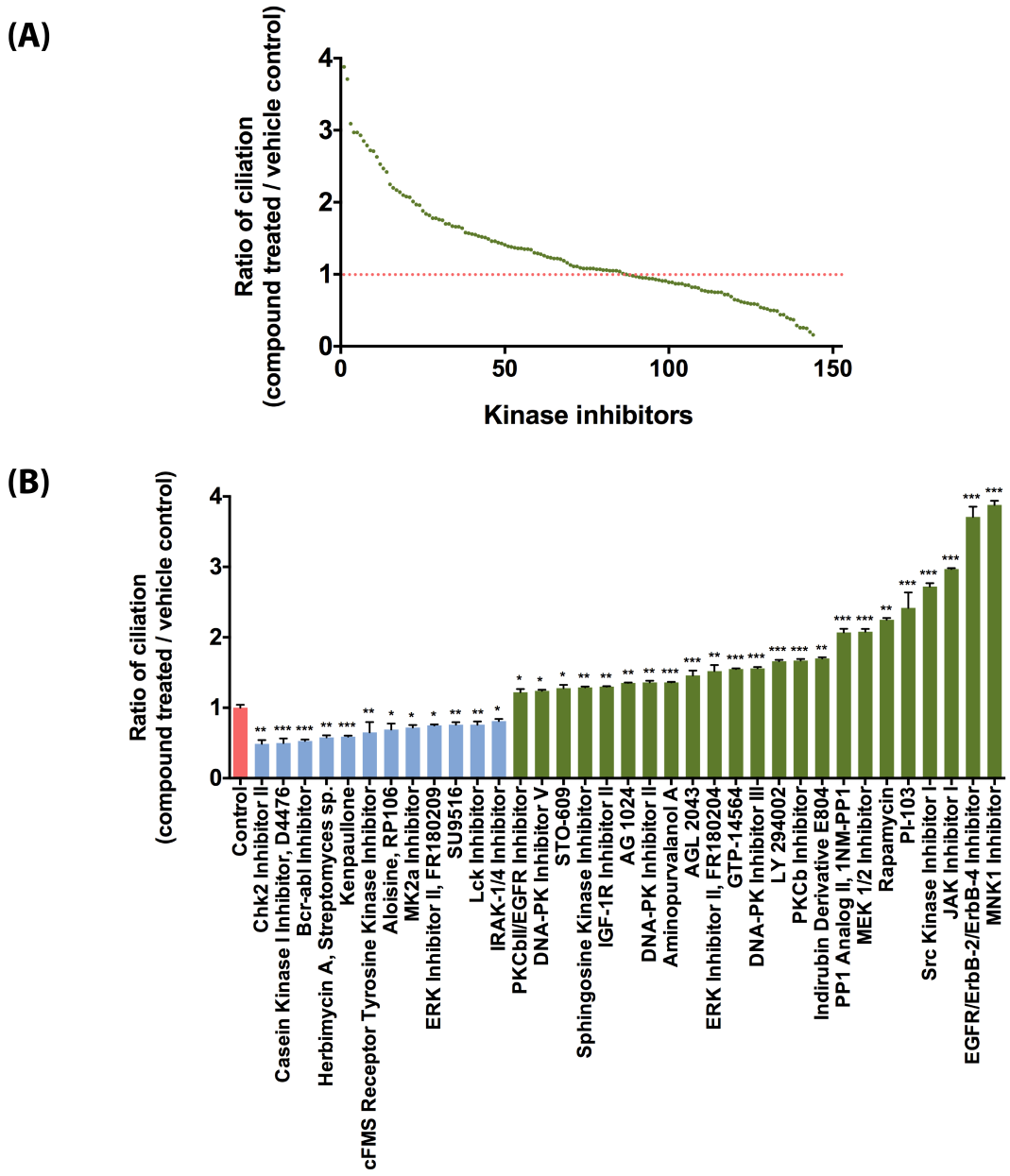

Figure 4.1. Identification of cilium modulators in a kinase inhibitor library using the IN **Cell Analyzer.** (A) Ciliogenic potential of 160 compounds of the kinase inhibitor library as revealed by assessing the percentage of ciliated CFPAC-1 cells (acetylated tubulin staining) using the IN Cell Analyzer 8 days after compound treatment (1 micromolar). Compounds are ranked in descending order of their potency to increase the percentage of ciliated cells relative to vehicle control (pink dotted line). (B) List of cilium suppressors (blue bars) and enhancers (green bars) based on the criterion to reduce the percentage of ciliated cells by more than 3 times the standard deviation as compared to control cells and ranked according to their effect. Data are presented as mean ± S.D. *p ≤ 0.05, **p ≤ 0.005, ***p ≤ 0.0005 as compared to control.

4.2. Results

4.2.1. Identification of cilium modulators from a compound screen of kinase inhibitors.

To identify potential modulators of ciliogenesis, we employed a 96-well format screening strategy previously developed by us [9], which evaluates the changes in percentage of ciliated cells in CFPAC-1 cells by immunofluorescence microscopy-based visualization of

primary cilia stained with an antibody specific to the ciliary component acetylated tubulin. Using this strategy we screened a library of 160 kinase inhibitors (Figure 4.1A). All compounds were screened in triplicate. Compounds (hits) were considered

Table 4.1. List of cilia-modulating kinase inhibitors identified from the library screen and their Unique numerical identifier numbers (CAS numbers).

INHIBITOR NAME	CAS NUMBER
Cilia suppressors	
Chk2 Inhibitor II	516480-79-8
Casein Kinase I Inhibitor, D4476	301836-43-1
Bcr-abl Inhibitor	778270-11-4
Herbimycin A, Streptomyces sp.	70563-58-5
Kenpaullone	142273-20-9
cFMS Receptor Tyrosine Kinase Inhibitor	870483-87-7
Aloisine, RP106	496864-15-4
MK2a Inhibitor	41179-33-3
ERK Inhibitor II, FR180209	1177970-73-8
SU9516	666837-93-0
Lck Inhibitor	213743-31-8
IRAK-1/4 Inhibitor	509093-47-4
Cilia inducers	
PKCbII/EGFR Inhibitor	145915-60-2
DNA-PK Inhibitor V	404009-46-7
STO-609	52029-86-4
Sphingosine Kinase Inhibitor	1177741-83-1
IGF-1R Inhibitor II	196868-63-0
AG 1024	65678-07-1
DNA-PK Inhibitor II	154447-35-5
Aminopurvalanol A	220792-57-4
AGL 2043	22617-28-8
ERK Inhibitor II, FR180204	865362-74-9
GTP-14564	34823-86-4
DNA-PK Inhibitor III	404009-40-1
LY 294002	154447-36-6
PKCb Inhibitor	257879-35-9
Indirubin Derivative E804	854171-35-0
PP1 Analog II, 1NM-PP1	221244-14-0
MEK 1/2 Inhibitor	305350-87-2
Rapamycin	53123-88-9
PI-103	371935-74-9
Src Kinase Inhibitor I	179248-59-0
JAK Inhibitor I	457081-03-7
EGFR/ErbB-2/ErbB-4 Inhibitor	881001-19-0
MNK1 Inhibitor	522629-08-9

as cilia suppressors or enhancers based on their ability to reduce or respectively increase the percentage of ciliated cells by more than 3 times the standard deviation as compared to control cells. The hits based on low cell count were considered unreliable and hence

excluded from further evaluation. Based on these criteria, 12 potential cilium suppressors and 23 cilium enhancers (Figure 4.1B) were identified from the initial screen. Table 4.1 shows the unique numerical identifier (CAS Number) of each identified hit.

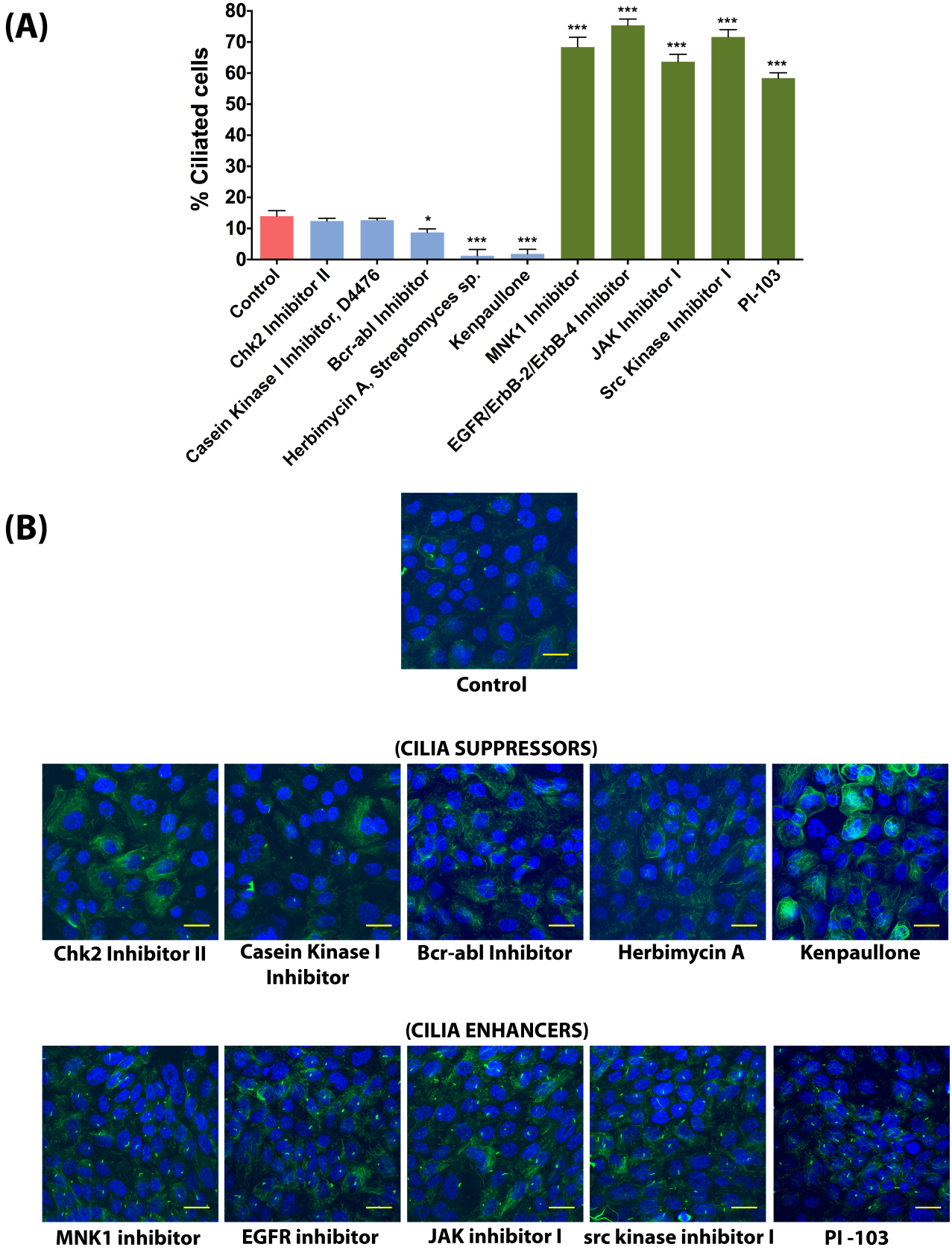

Figure 4.2. (on page 108) Reconfirmation of the cilium modulatory effect of the 10 best hits by confocal microscopy of CFPAC-1 cells. (A) Quantitative analysis of the effect of the best 5 cilium suppressors and enhancers from Figure 4.1 on the percentage of ciliation in CFPAC-1 cells as determined by confocal microscopy. 200-300 cells were counted from at least three different regions of the well to perform the quantification. All data are presented as mean ± SEM, *p ≤ 0.05, **p ≤ 0.005, ***p ≤ 0.0005 as compared to control. (B) Images of primary cilia in compound-treated CFPAC-1 cells as visualized by confocal microscopy. Cilia suppressors and enhancers are indicated by red and black font colors respectively. Cilia were stained with anti-acetylated tubulin antibody (green) and nuclei were counterstained with DAPI (blue). Scale bar represents 10 μm.

4.2.2. Confirmation of cilium modulation by confocal microscopy

Out of the 12 cilia suppressors and 23 cilia enhancers identified in the initial screen, we selected the 5 most potent suppressors from each category for confirmation of their cilio-modulatory effect by a more robust confocal microscopy technique, which allows the elimination of false positives. Three out of 5 potential repressors and all 5 selected enhancers significantly modulated cilium expression (Figure 4.2A and 4.2B). The two most potent suppressors (Herbimycin A, Kenpaullone) and enhancers (EGFR/ErbB-2/ErbB-4 inhibitor, Src Kinase Inhibitor I) of cilia were selected for further testing.

4.2.3. Herbimycin A and Kenpaullone suppress ciliogenesis and attenuate cell proliferation in cilium-dependent glioblastoma cells. We next evaluated the most potent cilia suppressors Herbimycin A and Kenpaullone for their ability to suppress cilia in a glioblastoma cell line model GL2664, the growth of which is dependent on the primary cilium and hence is richly ciliated in the basal condition (Figure 4.3). Both the compounds Herbimycin A and Kenpaullone significantly inhibited ciliogenesis in this cell line and reduced the percentage of ciliated cells from approximately 70% to 5% or less (Figure 4.3). This result highlights the potential of these compounds as potent cilium suppressors in highly ciliated and cilium-dependent cancer. Both compounds significantly attenuated the growth of the cilium-dependent GL2664 glioblastoma cells but not of the CFPAC-1 pancreatic cancer cells, which are relatively poorly ciliated and the growth of which is known to be repressed by cilium expression (Figure 4.4A). This result was further substantiated by measurement of cell proliferation based on BrdU incorporation in cells treated with the compounds. Both compounds significantly reduced BrdU incorporation in glioblastoma cells but not of pancreatic cancer cells (Figure 4.4B). As revealed by FACS analysis both compounds caused a shift in the percentage of cells in the G0-G1 phase to those in the G2-M phase of the cell cycle of

glioblastoma cells, whereas CFPAC-1 pancreatic cancer cells were unaffected (Figure 4.4C). These findings highlight the potential of Herbimycin A and Kenpaullone to attenuate the growth of glioblastoma cells that are dependent on cilia for their survival and growth.

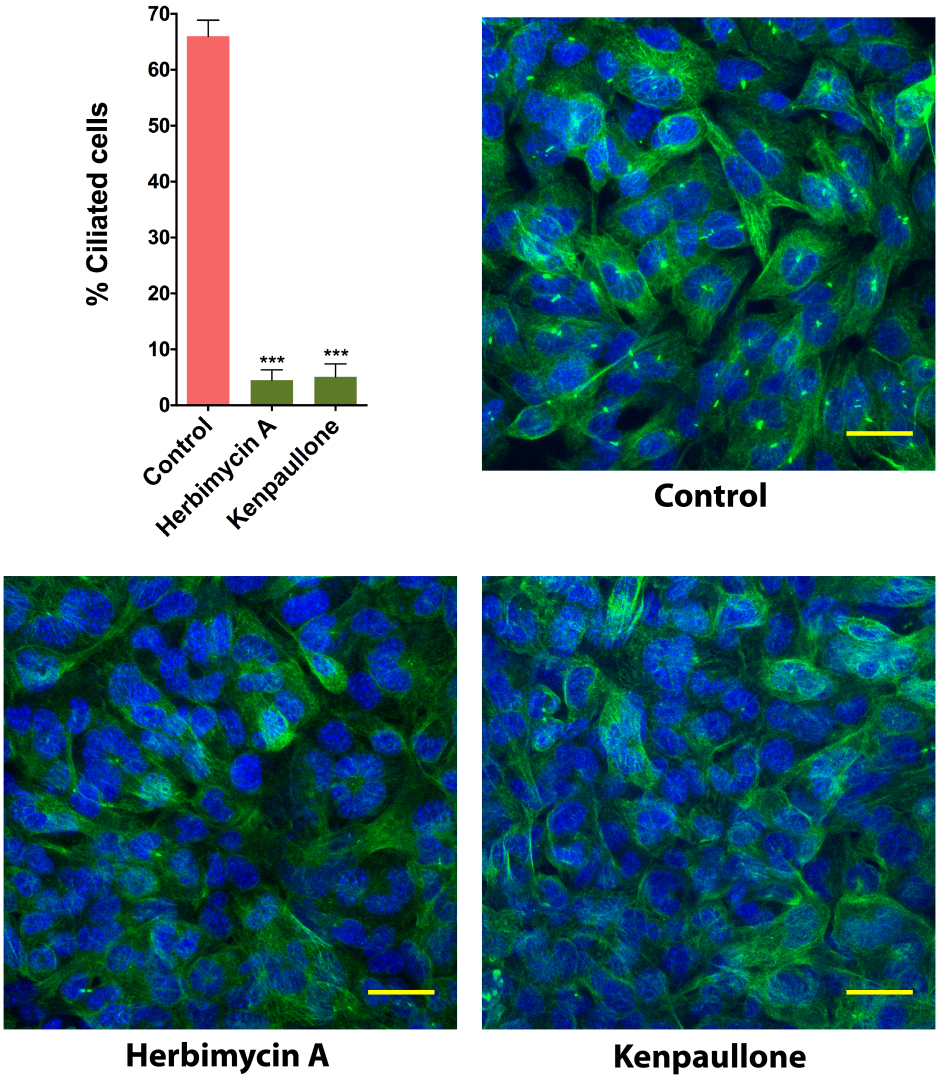

Figure 4.3. Effect of Herbimycin A and Kenpaullone on ciliogenesis in GL2664 glioblastoma cells. Quantification of the percentage of ciliated cells after treatment of cells with Herbimycin A and Kenpaullone. Data was obtained by counting 200-300 cells from three different regions of the well and is presented as mean ± SEM, *p ≤ 0.05, **p ≤ 0.005, ***p ≤ 0.0005 as compared to control. Representative confocal microscopy images show the effect of Herbimycin A and Kenpaullone treatment on glioblastoma cells. Scale bar in the images represents 10 µm.

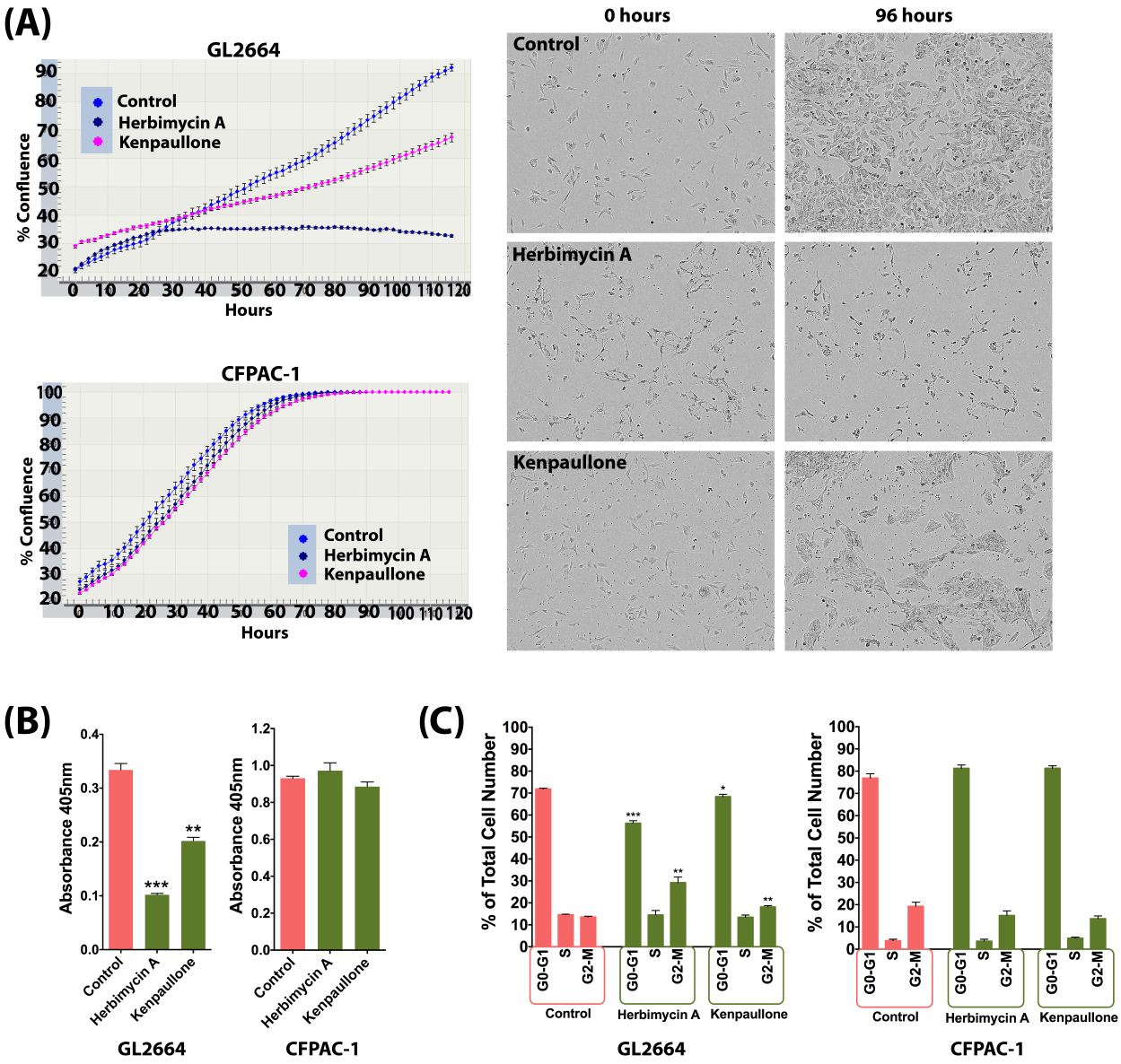

Figure 4.4. Effect of Herbimycin A and Kenpaullone on cell proliferation of cilium-dependent and cilium-independent cancer cell lines. (A) Effect of indicated compounds on the growth of GL2664 and CFPAC-1 cancer cells as assessed by Incucyte live cell imaging and analysis. Data was obtained by calculating the mean value of confluence from 6 different wells of each condition. The panel on the right shows representative images of control and compound-treated GL2664 cells as captured by Incucyte at different time points. (B) Effect of the indicated compounds on proliferation of GL2664 and CFPAC-1 cells as measured by BrdU incorporation assay. (C) Assessment of changes in cell cycle profile as determined by FACS analysis of GL2664 and CFPAC-1 cells treated with indicated compounds. Data are presented as mean ± SEM, *p ≤ 0.05, **p ≤ 0.005, ***p ≤ 0.0005 as compared to control.

4.2.4. EGFR/ErbB-2/ErbB-4 inhibitor and Src Kinase Inhibitor I attenuate cell proliferation in poorly ciliated pancreatic cancer cells

To assess the ability of cilia enhancers to inhibit the proliferation of other poorly ciliated cells that benefit from cilium loss, we treated CFPAC-1 pancreatic cancer cells with EGFR/ErbB-2/ErbB-4 inhibitor and Src Kinase Inhibitor I and measured cell proliferation

using the BrdU test. As shown in Figure 4.5, both inhibitors attenuated cell proliferation in CFPAC-1 cells. As revealed by FACS analysis, this was accompanied by a shift from cells in the G2-M phase to the G0-G1 phase (Figure 4.5A and 4.5B). We confirmed the anti-proliferative effect of these compounds in another pancreatic cancer cell line model, L3.6 pancreatic cancer cells, which can be grown as spheroids to better mimic the growth of naturally occurring human tumors [9]. Both EGFR/ErbB-2/ErbB-4 inhibitor and Src Kinase Inhibitor I were effective in reducing the spheroid volume as compared to controls (Figure 4.5C).

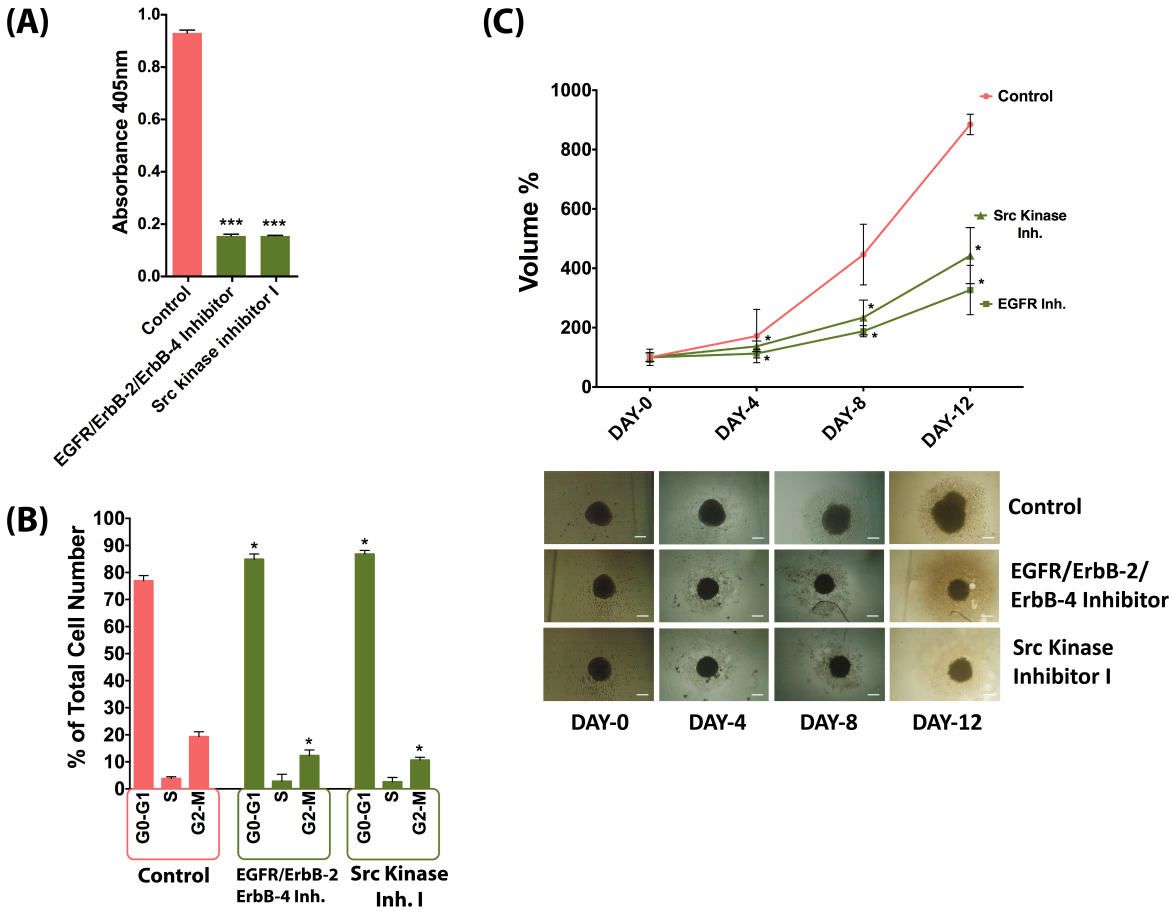

Figure 4.5. Effect of cilium enhancers EGFR/ErbB-2/ErbB-4 inhibitor and Src Kinase Inhibitor I on cell proliferation of poorly ciliated pancreatic cancer cells. (A) Effect of indicated compounds on proliferation of CFPAC-1 pancreatic cancer cells as assessed by BrdU incorporation assay. (B) Assessment of cell cycle profile by FACS analysis of CFPAC-1 cells treated with the indicated compounds. Data are presented as mean ± SEM, *p ≤ 0.05, **p ≤ 0.005, ***p ≤ 0.0005 as compared to control. (C) Effect of indicated compounds on the growth of L3.6 pancreatic cancer spheroids. The volume was calculated as a mean of at least 6 spheroids for each condition. Lower panel displays representative images of untreated and treated spheroids at different time intervals.

4.3. Discussion

Venturing into the hitherto unexplored area of cancer therapy based on cilium modulation, we have screened a library of 160 kinase inhibitors for potential modulators of ciliogenesis

and identified two classes of compounds: cilium suppressors and cilium enhancers. A deeper look into the molecular targets and modes of action of the identified cilium suppressors and inducers provides interesting clues into some of the possible mechanisms that might be involved in their differential effect on ciliogenesis. Herbimycin A is a pleiotrphic inhibitor that among others inhibits Hsp90 [10, 11], which is a heat shock protein that is associated with microtubules of the cilium and is important for the stabilization of tubulin [12, 13]. Hence, the suppression of ciliogenesis by Herbimycin A might in part be attributed to the destabilization of microtubules potentially involving an HDAC6-HSP90 interplay [14]. Kenpaullone is an inhibitor of GSK-3β, an important protein involved in the maintenance of cilia [15, 16]. It also inhibits several CDKs, suggesting that Kenpaullone may also suppress ciliogenesis by the regulation of the cell cycle. Interestingly, both compounds cause a shift in the percentage of cells in the G0-G1 phase, which is typically associated with cilium expression, towards those in the G2-M phase, which usually are devoid of cilia as the structural elements of the cilium are used for chromosome segregation. To what extent changes in cell cycle are cause or consequence or work hand-in-hand with alterations in ciliogenesis remains unknown.

With respect to cilia enhancers, the ciliogenic effect of EGFR/ErbB-2/ErbB-4 Inhibitor is likely attributed to its ability to inhibit EGFR, which is known to activate MAPK signaling, a negative regulator of ciliogenesis [17, 18]. Therefore, it is plausible that suppression of hyperactive MAPK signaling through inhibition of EGFR activity leads to the enhancement of ciliogenesis. Interestingly, it has also been shown that EGFR is a part of protein complexes that are involved in the localization of a sensory protein to the cilium [19]. However, it is not yet known if it is also involved in the localization of structural proteins that are required for the formation of cilium. Src Kinase Inhibitor I is known to directly bind and phosphorylate NEDD9, a protein involved in the inhibition of ciliogenesis [20, 21]. The activated NEDD9 in turn activates HDAC6 via AURKA to inhibit ciliogenesis [22]. Thus, inhibition of Src Kinase activity may promote ciliogenesis by inhibiting NEDD9-mediated disassembly of the cilium. Previous studies have show that Src Kinase inhibition has therapeutic effects in polycystic kidney disease [23, 24]. Recently, a study has shown that an actin regulatory protein MIM (Missing-in Metastasis) is present at the basal body and promotes ciliogenesis by antagonizing src-dependent activation of another protein cortactin [25]. Therefore, inhibition of Src Kinase may promote ciliogenesis by positive regulation of MIM.

In view of earlier findings that various cancer types show distinct and opposite dependencies and growth (dis)advantages linked to the primary cilium [7, 8], the two classes of compounds may find other applications depending on the tumor type (Figure 4.6). Cilium inhibitors may have therapeutic potential mainly in cilium-dependent cancers like glioblastomas, basal cell carcinomas, medulloblastomas, whereas cilium enhancers could be used to treat cancers like pancreatic, breast, prostate cancers and melanomas that benefit from the loss of the cilium (Figure 4.6). Our findings may also have broader implications as they can also be expanded to develop therapeutic strategies for the treatment of other cilium-related disorders and ciliopathies.

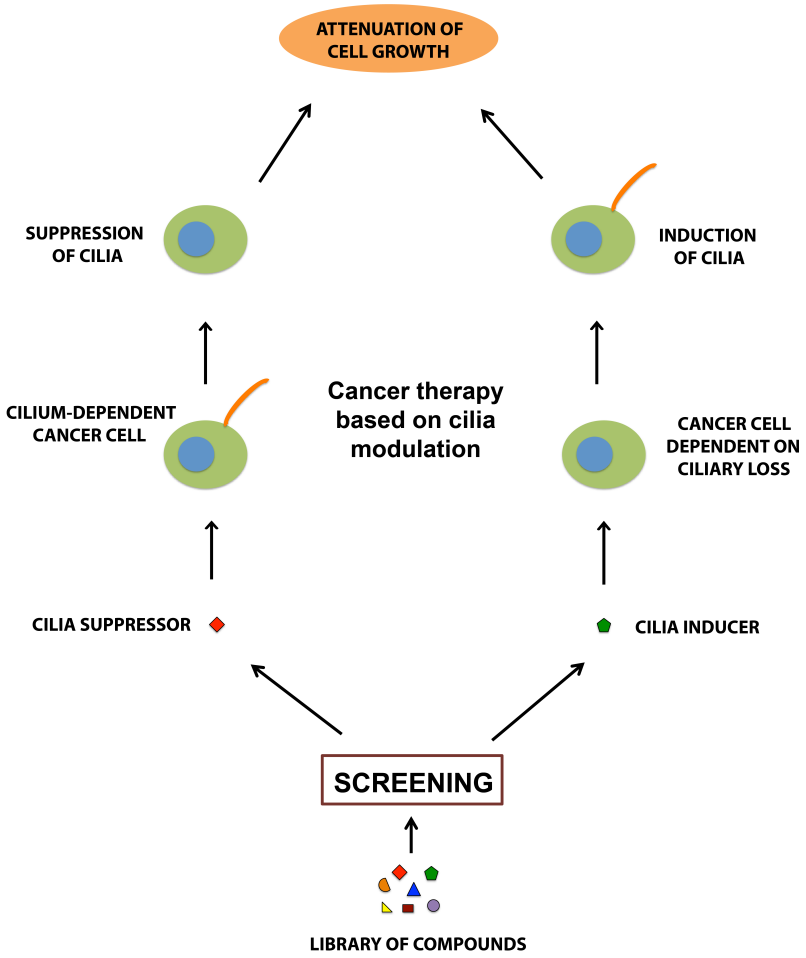

Figure 4.6. Overview of the therapeutic strategy to treat cancers with different cilium dependencies. Two classes of compounds were identified in our screen: cilium suppressors and cilium enhancers. Cilium suppressors can be used as potential therapeutic agents against cancers that are dependent on the cilium, whereas cilia inducers can be used as therapeutics against cancer cells that are dependent on the loss of the cilium.

4.4. Materials and methods

4.4.1. Cell culture

CFPAC-1 and L3.6 pancreatic cancer cell lines were obtained from ATCC. The GL2664 glioblastoma cell line was attained from the laboratory of Angiogenesis and

Neurovascular Link (KU Leuven, Belgium). CFPAC-1 and L3.6 cells were maintained in RPMI-1640 medium (Life Technologies) supplemented with 10% FBS (Life Technologies). The medium for L3.6 cells was supplemented with 4mM L-Glutamine (Life Technologies). GL2664 cells were cultured in DMEM high glucose medium (Life Technologies) supplemented with 10% FBS and 4mM L-Glutamine. All the cell lines were grown at a temperature of 37°C in a humidified incubator with 5% levels of CO_2.

4.4.2. Screening of protein kinase inhibitor library

A library comprising of a panel of 160 well- characterized, cell permeable, potent and reversible protein kinase inhibitors (Calbiochem) was procured from Center for Drug Design and discovery (CD3) (Leuven, Belgium). The compounds were dissolved in DMSO and stored as 10mM stock solutions before addition to culture medium at a final concentration of 1 μM. The library was screened according to the methodology developed by us previously [9] based on CFPAC-1 pancreatic cancer cells grown in 96-well format followed by immunofluorescence based imaging and analysis using IN Cell Analyzer and IN Cell Developer respectively, to assess changes in the percentage of ciliated cells by staining of cilium with anti-acetylated tubulin antibody (Sigma, Cat No. T6793-.5ML) followed by a fluorescent secondary antibody (Life Technologies, AlexaFluor 488, Cat No. A21145).

4.4.3. Immunofluorescence confocal microscopy

CFPAC-1 and GL2664 cells were seeded on glass coverslips placed in 12-well plates containing 1ml of culture medium per well. When cells were fully confluent, the culture medium was replaced with fresh medium containing 2% FBS and treated with compounds for 24 hours and fixed with 4% Formaldehyde (Merck), permeabilized with 0.1% Triton X100 (Merck) in DPBS, blocked with 1% BSA (Applichem) in DPBS, incubated with anti-acetylated tubulin antibody (1:1000) dilution for 1 hour, followed by incubation with a fluorescent secondary antibody for 1 hour. Nuclei were stained with DAPI (Vector Laboratories, Vectashield, Cat. No. H-1500). Nikon C2 Eclipse Ti-E confocal laser scanning microscope was used to capture images of cilia as Z-stacks using a 60X oil immersion lens. Compounds selected for confirmation experiments and functional assays were purchased from Calbiochem.

4.4.4. Cell growth assay

Cell growth assays were performed in 96-well plates for a period of 5 days using Incucyte (Essen Bioscience), a live cell imaging system that captures bright phase images at an interval of every 2 hours from 4 separate regions per well using a 20X lens. Assay plates were maintained at 37°C in an incubation chamber throughout the run. The Incucyte software was used to plot the growth curves by calculating mean confluence from six wells of the 96-well plate for each condition.

4.4.5. BrdU incorporation assay:

BrdU incorporation assay to measure cell proliferation was performed using 5-Bromo-2-deoxy-uridine Labeling and Detection Kit III (Cat. No. 11444611001, Roche) following the manufacturer protocol.

4.4.6. Tumor spheroid assay: Tumor spheroid assay based on L3.6 pancreatic cancer cells was performed as described in [9].

4.4.7. Cell cycle analysis

GL2664 and CFPAC-1 cells were plated in 6-well plates at a density of 300,000 cells/well and allowed to reach 60% confluence before the start of compound treatment for 48 hours. Post treatment, cells were harvested by trypsinization, fixed with 4% Formaldehyde, permeabilized with 0.1% Triton X100, followed by staining with Vybrant DyeCycle Green Stain (Cat. No. V35004, ThermoFisher Scientific). Data was obtained using BD FACSCanto Flow Cytometer (Becton Dickinson) by measuring at least 10,000 events per sample. Data was analyzed using FlowJo software (Becton Dickinson).

4.4.8. Statistical analysis

Statistical analysis was performed using GraphPad Prism version 6 for Mac OS X (GraphPad Software, San Diego, California, USA). Data are expressed as mean ±S.D. or ±SEM. Differences between two groups were calculated using the t-test. Statistical significance was set at p value of < 0.05.

4.5. References

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CHAPTER 5

Identification and functional testing of novel small molecule compounds that reverse SREBP1-mediated suppression of the primary cilium: application to models of cancer and ciliopathies

Identification and functional testing of novel small molecule compounds that reverse SREBP1-mediated suppression of the primary cilium: application to models of cancer and ciliopathies

5.1. Introduction

The primary cilium is a single, slender eyelash-like structure that grows at the apical surface of the cell. It is a microtubule-based organelle that is present on almost every cell type in mammals. Primary cilia function as sensors of extracellular signals. They capture signals from their immediate surroundings and transduce them into the cell, which is then informed of how to adapt and develop itself. Thus, a primary cilium acts as an antenna that serves as a communication device between the cell and the extracellular environment. To this end a large diversity of receptors, proteins, or channels can be present on the same cilium. Many different signal transduction systems may exist on a single primary cilium to facilitate diverse signaling processes that are instrumental in tissue development. These include Hedgehog, Wnt and PDGFRα pathways that are pivotal for embryonic growth and development.

Being a hub of cellular signaling, structural integrity of the cilium and ciliary components becomes cardinally important. Abrogation of the cilium or loss of ciliary function leads to profound effects that compromise normal development and health of an organism [1]. Ciliary dysfunction results in specific disorders called ciliopathies that include Polycystic Kidney Disease (PKD), Primary Cilia Dyskinesia (PCD), Bardet-Biedl Syndrome (BBS), Joubert Syndrome (JBTS), Meckel Syndrome (MKS), Kartagener Syndrome etc. Interestingly, there is increasing evidence that the primary cilium is also involved in other much more common human diseases like cancer [2]. In fact, emerging evidence implicates a decrease, loss, distortion or ablation of primary cilia in a number of cancers [3-5] like prostate cancer, pancreatic cancer, breast cancer, melanoma etc. Although the exact mechanisms underlying cilium loss in cancer development remain unknown, evidence from our team has shown that aberrant lipid metabolism and particularly constitutive activation of the lipogenic transcription factor SREBP1 may play an important role in cilium repression [6]. As to the role of cilium loss in cancer

cells, it is commonly assumed that the cilium puts a break on cell proliferation as it uses the same structural components as required for chromosome segregation. Moreover, cilium loss may contribute to distorted cellular signaling that is a hallmark of cancer [7-9]. Hence, the primary cilium and particularly the mechanisms that govern primary ciliogenesis may be interesting novel targets for antineoplastic therapy.

In the present study, we have developed a chemical screening platform based on SREBP1c-mediated cilium suppression in MDCK kidney cells to identify novel compounds that can revive primary cilium in cancer cells. This screening method was further applied to screen a library of 1300 small molecules, which revealed 8 compounds that had the ability to potently induce cilia in several cancer models and to rescue ciliary length in a ciliopathy model. By virtue of their ability to induce cilia, these compounds attenuated cell proliferation in melanoma, breast and prostate cancer models. These findings provide a solid groundwork to support the feasibility of using a new concept of cilium restoration as a strategy to treat cancers and ciliopathies.

5.2. Results

5.2.1. Development of a screening strategy based on normal kidney cells with forced SREBP-induced cilium repression

In view of our previous studies showing that aberrant activation of the lipogenic transcription factor SREBP1 mediates primary cilium repression in various models [6], we developed a compound screening approach using normal kidney cells (MDCK) transduced with an SREBP1c gene-encoding adenovirus to identify chemical compounds with the ability to re-induce the cilium. Transduction, seeding and culture conditions were thoroughly optimized for consistency in cilium repression (down to 20% ciliated cells in the culture) and formation of flat monolayers amenable for automated image acquisition by high content analysis using the IN Cell Analyzer. The acquired images were analyzed by the IN Cell Developer software followed by statistical analysis to identify the compounds that were able to reverse the SREBP-induced repression of primary cilium. Figure 5.1 illustrates the schematic workflow of the screening process. Using this model we screened a library of 1300 unknown compounds (CISTIM Library). This library was composed of compounds that were non-cytotoxic and easily soluble in aqueous solutions. Initially 102 hits were selected based their ability to increase the percentage of ciliated cells by at least 3-fold the standard deviation compared to vehicle-

treated cells. These compounds were rescreened in a second round using the same approach. This yielded 20 hits, the identity of which is kept undisclosed for reasons of intellectual property protection.

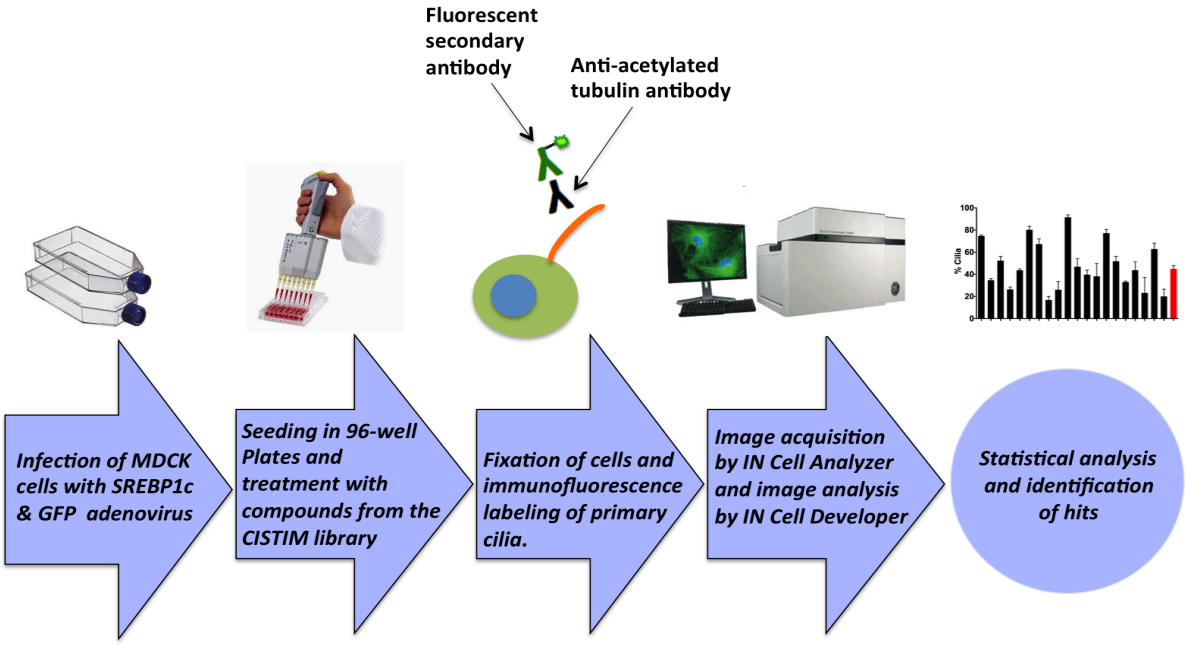

Figure 5.1. A schematic overview of the screening strategy of the CISTIM library using the adSREBP1c-transduced MDCK cell line model.

5.2.2. Confirmation of 8 ciliogenic compounds from the CISTIM Library by confocal fluorescence microscopy

The 20 hits from the InCell Analyzer screen were tested for their ability to induce cilium expression in LNCaP cells, a prostate cancer cell-line that is lipogenic and poorly ciliated (Figure 5.2A). 8 of the 20 compounds potently induced ciliogenesis and were selected for further studies (Figure 5.2A). Since we cannot reveal the identity of these compounds, we labeled them C1 to C8. All 8 of these compounds were reconfirmed by confocal microscopy to re-induce cilium expression in adSREBP1c-transduced MDCK cells (Figure 5.2B and 5.2C). All compounds increased the percentage of ciliated cells by more than 2-fold as compared to untreated control. The ability of these compounds to reverse the SREBP1c-mediated repression of ciliogenesis marks their prospective use as possible re-inducers of the primary cilium in lipogenic cancers.

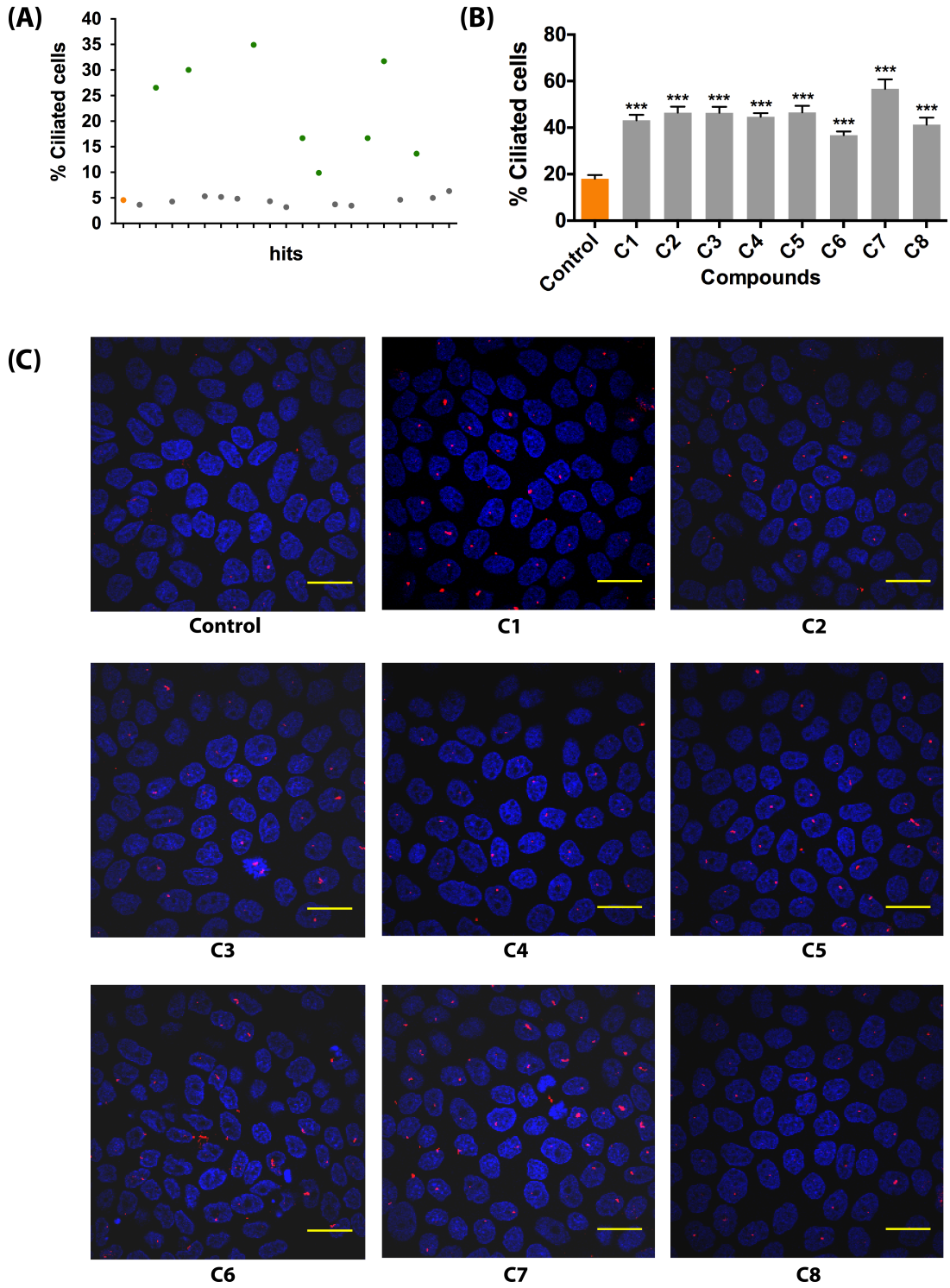

Figure 5.2. Selection of the 8 most potent ciliogenic compounds and reconfirmation in adSREBP1c-transduced MDCK cells by confocal microscopy. (A) Scatter diagram showing the ability of compounds to increase the percentage of ciliated cells in LNCaP cultures. The orange dot indicates the % of ciliated cells in untreated control cultures. Green dots indicate compounds selected for further analysis. (B) MDCK cells were infected with SREBP1c adenovirus (adSREBP1c) and treated with the compounds selected in (A) for 8 days. The percentage of ciliated cells was quantified by counting 100-300 cells from at least three regions of the well using ImageJ software. Data are presented as mean ± SEM, *p ≤ 0.05, **p ≤ 0.005, ***p ≤ 0.0005 as compared to control. (C) Confocal fluorescence microscopy images of primary cilia in adSREBP1c-transduced MDCK cells treated with the compounds selected in (A). Cilia were stained using an antibody against acetylated tubulin (red) and nuclei were stained with DAPI (blue). Images were captured using a Bio-Rad Radiance confocal microscope through a 40X objective lens at 2.3X zoom. The scale bar represents 5 µm.

5.2.3. Confirmation of cilium induction in multiple lipogenic cancer cell line models

These 8 selected compounds were further tested for their ability to induce ciliogenesis in several other lipogenic cancer cell lines: WM2664 (melanoma), SKBR3 (breast cancer), A549 (lung cancer), which all show an inherently low expression of ciliogenesis even at high confluency (2-12 % of the cell population). The compounds

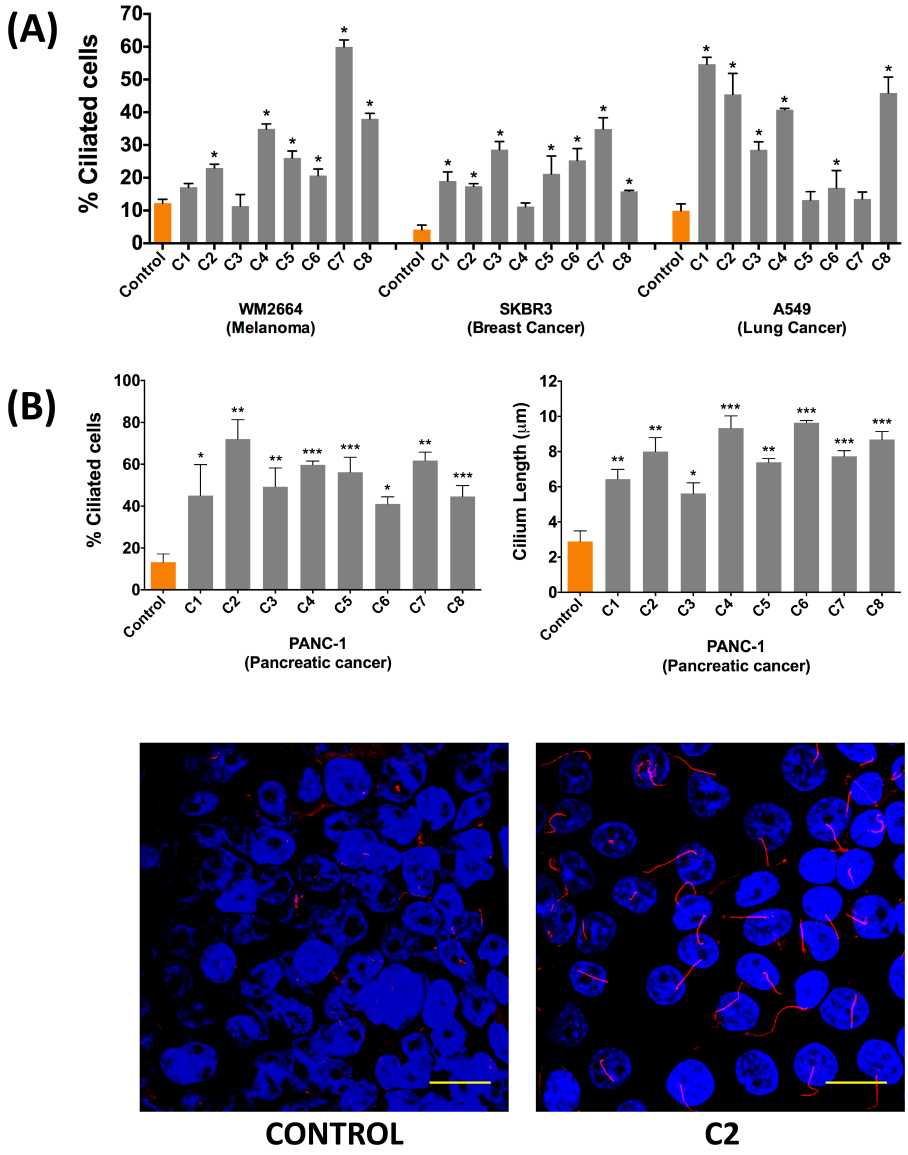

Figure 5.3. Determination of the ciliogenic potential of selected compounds in different lipogenic cancer cell line models by confocal microscopy. (A) Quantification of the percentage of ciliated cancer cells in various cancer cell lines treated with ciliogenic compounds for 8 days. (B) Graphs showing the effect of compounds on the percentage of ciliated cells and cilium length in the pancreatic cancer cell line model Panc1. Both the percentage of ciliated cells and changes in cilium length were analyzed and quantified by using ImageJ software. All data are presented as mean (n=100–300 cells) ± SEM, *p ≤ 0.05, **p ≤ 0.005, ***p ≤ 0.0005 as compared to control. Representative images in the lower panel show the effect of compound C2 on cilium induction in Panc1 cells. Cilia are stained in red (acetylated tubulin) and nuclei (DAPI) in blue. The scale bar represents 10 μm.

potently induced ciliogenesis in these models, with discernable cilia in up to 60% of the cell population (Figure 5.3A). The relative potency of the various compounds varied, however, from cell line to cell line. In addition to their ability to significantly increase the percentage of ciliated cells, the compounds were also able to increase the average cilium length in another lipogenic cancer cell-line PANC-1 (pancreatic cancer), which is an attractive model to assess changes in terms of both percentage of ciliated cells and cilium length (Figure 5.3B). Up to a 5-fold increase was observed in the percentage of ciliated cells whereas the average cilium length was enhanced by up to 3-fold by the ciliogenic compounds.

5.2.4. Selected compounds rescue cilia in 1p36 ciliopathy models.

To test whether the selected compounds were able to restore cilia also in ciliopathy models, we used a ciliopathy model (1p36 ciliopathy) that was developed by the team of Prof. W. Annaert in the context of a joint GOA project with our team. 1p36 is a ciliopathy caused by a deletion in the distal band of chromosome 1 resulting in the loss of Rer1p gene. Fibroblasts from 1p36 patients show a markedly shortened cilium length that has been linked with several ciliopathy features [12]. This effect was mimicked in LLC-CL4 (Pig epithelial cells) in which the Rer1p gene was knocked down using a siRNA approach. Loss of Rer1p led to the shortening of the cilium similar to that seen in patients and in patient-derived fibroblasts. As illustrated in Fig 5.4A, all compounds tested were able to fully rescue the cilium length in Rer1p knockdown cells. As shown for compound C2 in Fig 5.4B, also in primary fibroblasts from 1P36 patients, treatment with the compound resulted in a significant increase in the length of the cilia. These experiments were carried out in collaboration with the team of Prof. Wim Annaert.

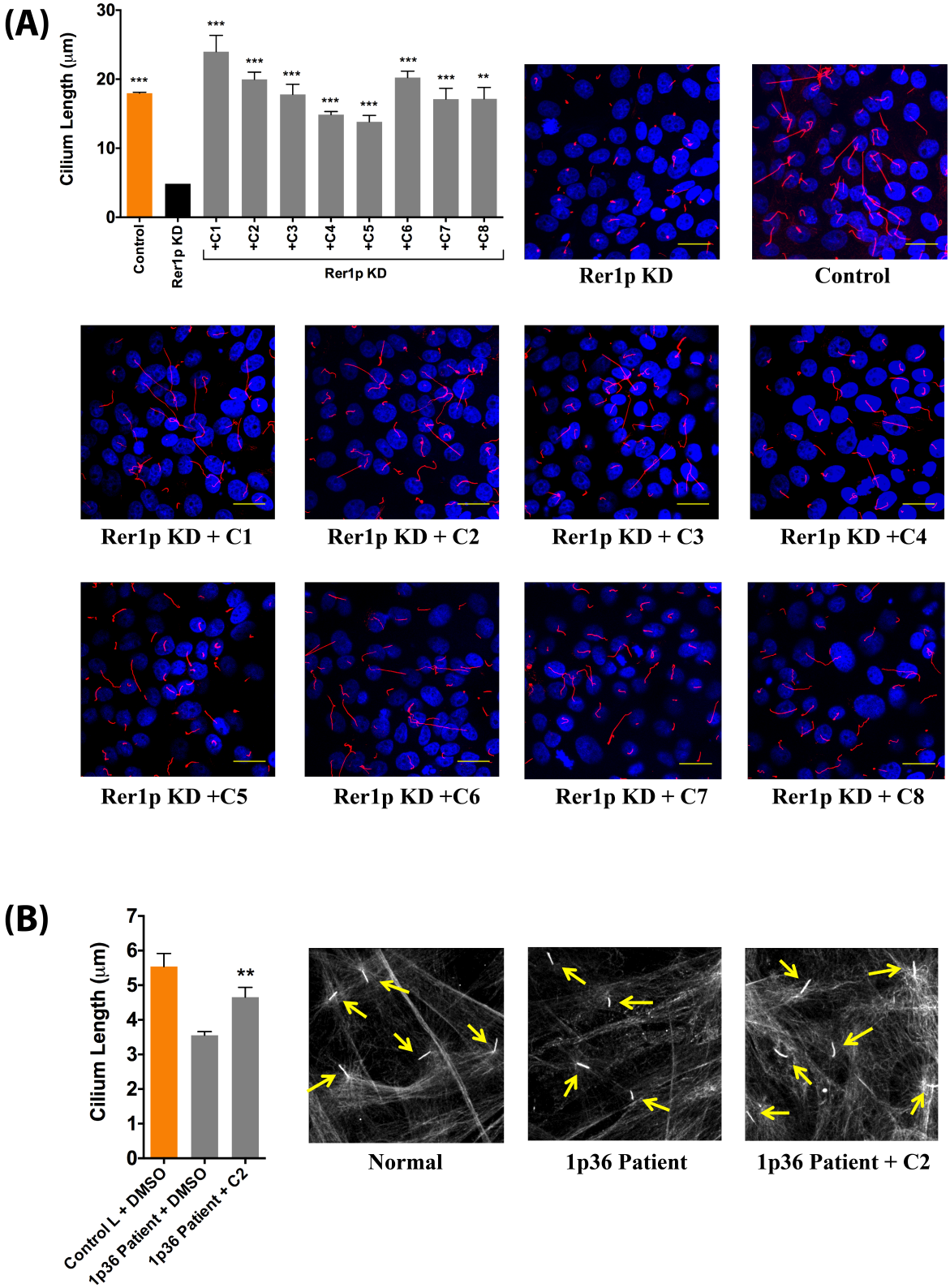

Figure 5.4. Rescue of primary cilia by ciliogenic compounds in 1p36 ciliopathy models. (A) LLC-CL4 cells in with the Rer1p protein was knocked down using siRNAs was used a 1p36 ciliopathy model and was treated with selected compounds to assess their ability to restore the cilium length as assessed by immunofluorescent confocal microscopy with antibodies against acetylated tubulin to reveal cilia (red) and DAPI to stain nuclei (blue). (B) 1p36 patient fibroblasts were treated with compound 2 and cilia were stained as in A (yellow arrows). N=3, Error Bars = +/- SEM, P-value: * = ≤ 0.05, ** = ≤ 0.005, *** = ≤ 0.0005. The scale bar represents 10 μm.

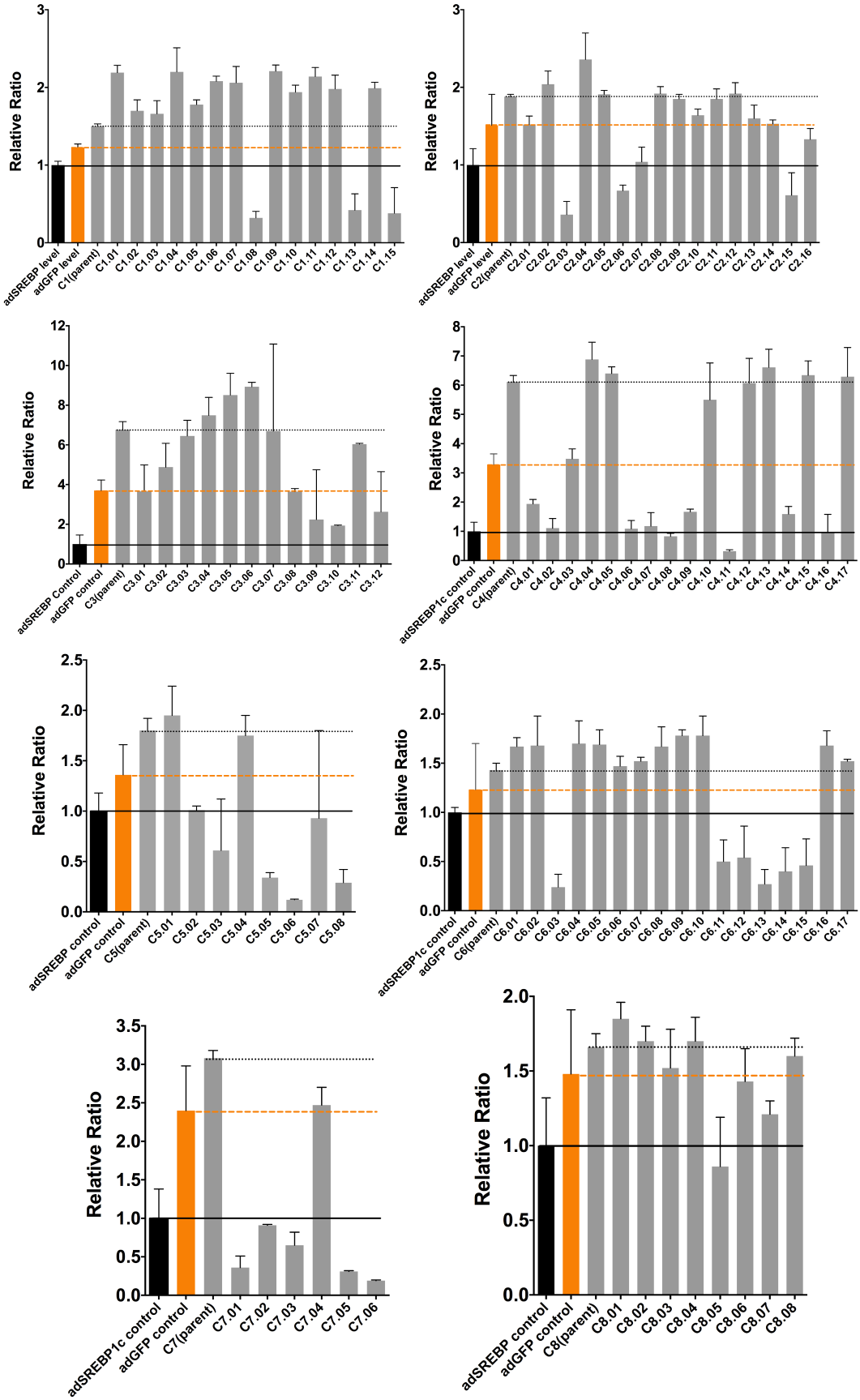

Figure 5.5. Structure Activity Relationship (SAR) assay for each family of ciliogenic compounds. MDCK cells were transduced with SREBP1c adenovirus (AdSREBP1c) (black bar) or with GFP control adenovirus (adGFP) (orange bar). AdSREBP1c cells were treated with equal concentrations of parent compounds and their analogs (grey bars). After 8 days, cilia were stained and quantified as in Figure 1. Changes in ciliation are expressed as relative ratio of ciliation with respect to untreated adSREBP control. The solid black line represents the percentage of ciliated cells in untreated AdSREBP1c cells and was set at 1. The dotted orange line represents the relative ciliation in AdGFP cells and the thin black dotted line represents the level of ciliation achieved by the parent compound in each family of compounds.

5.2.5. Structure Activity Relationship (SAR) studies reveal analogs that are better inducers of ciliogenesis as compared to respective parent compounds

Structure Activity relationship (SAR) is an approach to study the relationship between the molecular structure of a compound and it's biological activity. This study is critical to identify analogs with greatest potency and minimum side effects, and to establish which features of the molecular structure are important for its function. We have tested a panel of analogs for every compound and identified the most potent inducers of ciliogenesis. For compounds C2, C5 and C7, the original parent compounds picked from the library screen were found to be most potent (Figure 5.5). For the remaining compounds, the following analogs were identified as the most potent cilia inducers: analog 1.01 of compound C1, analog 3.06 of compound C3, analog 4.04 of compound C4, analog 6.10 of compound C6 and analog 8.01 of compound C8 (Figure 5.5). We also identified compounds that were less active than the parent compounds. This provides important information on the functional aspects of the structures. Analogs that were inactive and hence least potent in terms of induction of ciliogenesis were analogs 1.08, 2.03, 3.10, 4.11, 5.06, 6.03, 7.06 and 8.05 for parent compounds C1 to C8 respectively (Figure 5.5).

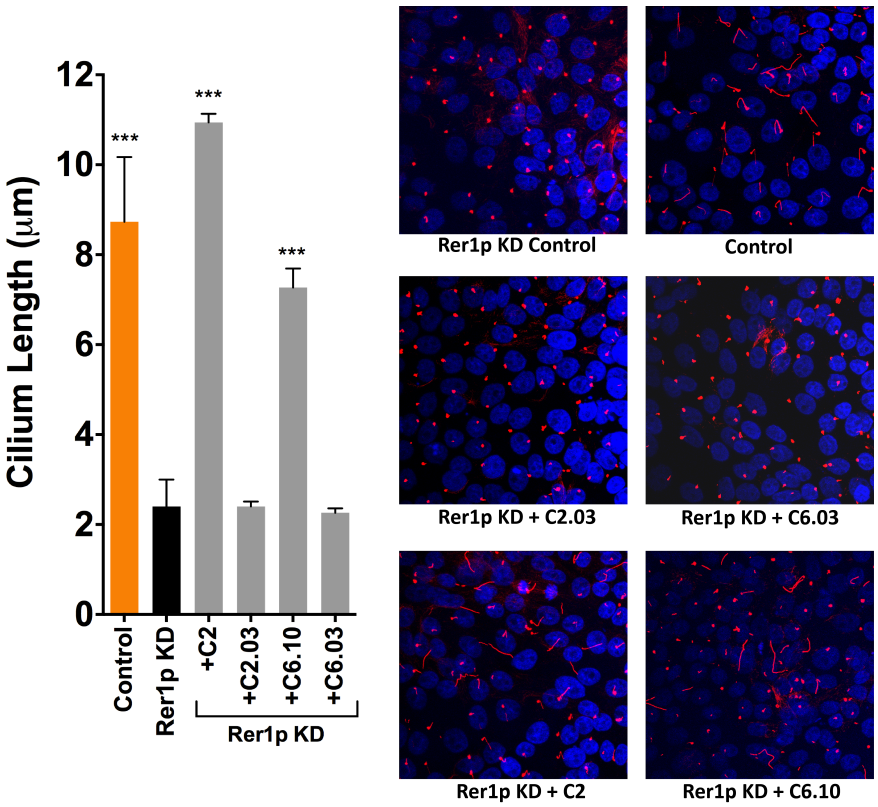

Figure 5.6. Comparative assessment of the ability of active and inactive analogs to rescue cilia in a ciliopathy model. Effect of the selection of analogs on rescue of ciliary length in LLC-CL4 Rer1p knockdown (KD) model. The panel on the right shows representative images of LLC-CL4 cells treated with the analogs. Cilia are stained in red and nuclei in blue. Data are presented as mean (n=100-300 cells) ± SEM, *p ≤ 0.05, **p ≤ 0.005, ***p ≤ 0.0005 as compared to control.

Next, we compared the ability of these ciliogenic and corresponding inactive analogs to rescue primary cilia in the previously described Rer1p knockdown 1p36 ciliopathy model. As expected, analogs C2 and C6.10 cause a resurgence of cilia in LLC-CL4 cells that have shorter cilia due to knockdown of Rer1p gene (Figure 5.6). As opposed to the active analogs, neither of the inactive analogs C2.03 or C6.03 was able to rescue the cilia in these cells (Figure 5.6). Pairs of active and inactive compounds are interesting tools for further functional studies.

5.2.6. Ciliogenic compounds reduce the proliferative potential of cancer cells

To test whether ciliogenic compounds affect the proliferation of cancer cells, we comparatively assessed the activity of ciliogenic analogs with their inactive

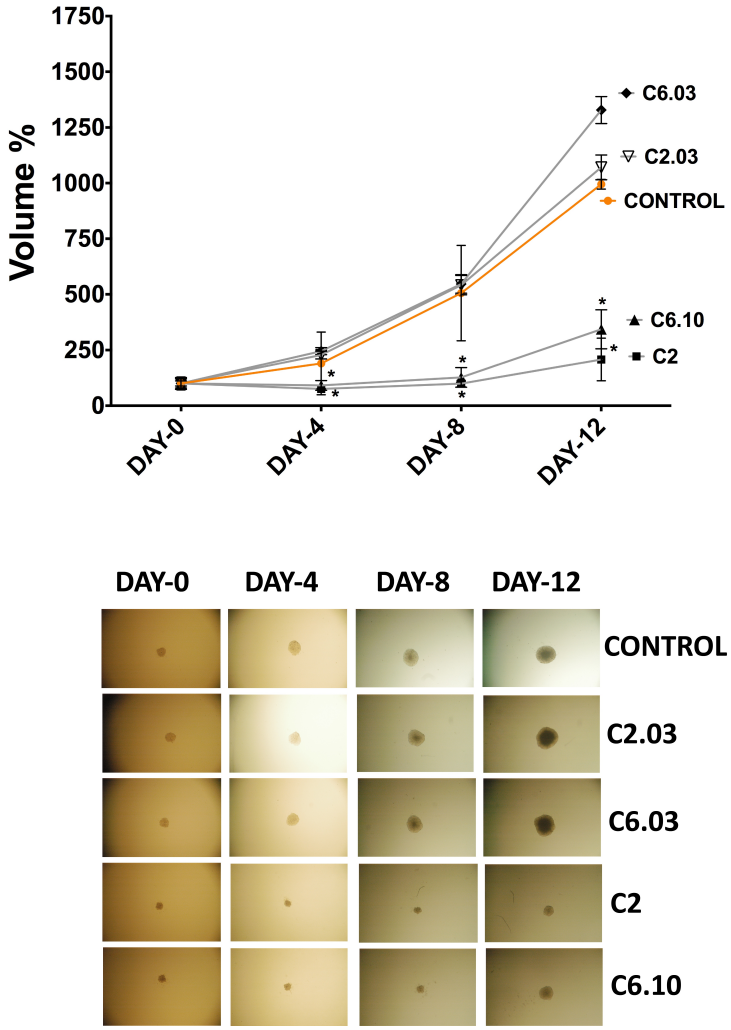

Figure 5.7. Effect of ciliogenic compounds and their inactive counterparts on the proliferative potential of cancer cells. A selection of active and inactive analogs were comparatively assessed on spheroid formation of LNCaP prostate cancer cells. Data are presented as the mean of at least 6 spheroids ± SEM, *p < 0.0005 as compared to control. The lower panel shows representative images of spheroids treated with vehicle control and with the indicated analogs

counterparts on the proliferative ability of LNCaP (Prostate cancer) cells grown as spheroids, which allows the monitoring of growth over a longer period of time and in a condition that more closely reflects the in vivo situation. As observed in Figure 5.7, ciliogenic analogs C2 and C6.10 significantly reduced spheroid growth, which was measured as the percentage increase in spheroid volume. In contrast, their respective inactive analogs C2.03 and C6.03 failed to reduce proliferation (Figure 5.7). This result indicates that reduction in spheroid growth is associated with the ability of the compound to induce the primary cilium in cancer cells.

5.2.7. Ciliogenic compounds stimulate Rab11 vesicle trafficking to the primary cilium

To further assess how ciliogenic compounds induce ciliogenesis, we explored the changes in localization of the endosomal-recycling marker Rab11 in pancreatic cancer cell line PANC-1. Rab11-positive vesicles have been shown to play an important role in the delivering of cargo to the growing primary cilium [13, 14]. In previous studies, our team has shown that distortion of ciliogenesis by SREBP1 is accompanied by a marked dispersion of Rab11 positive vesicles away from the base of the cilium. Figure 5.8 confirms that Rab11 is diffusely dispersed in the cytoplasm of poorly ciliated control cells. Treatment with the inactive analog C2.03 does not affect Rab11 distribution. However, in cells treated with ciliogenic compound C2, Rab11 is redirected to the base of the cilium. This result shows that ciliogenesis, at least by this compound C2 is accompanied by a normalization of Rab11-mediated vesicle trafficking to the base of the cilium.

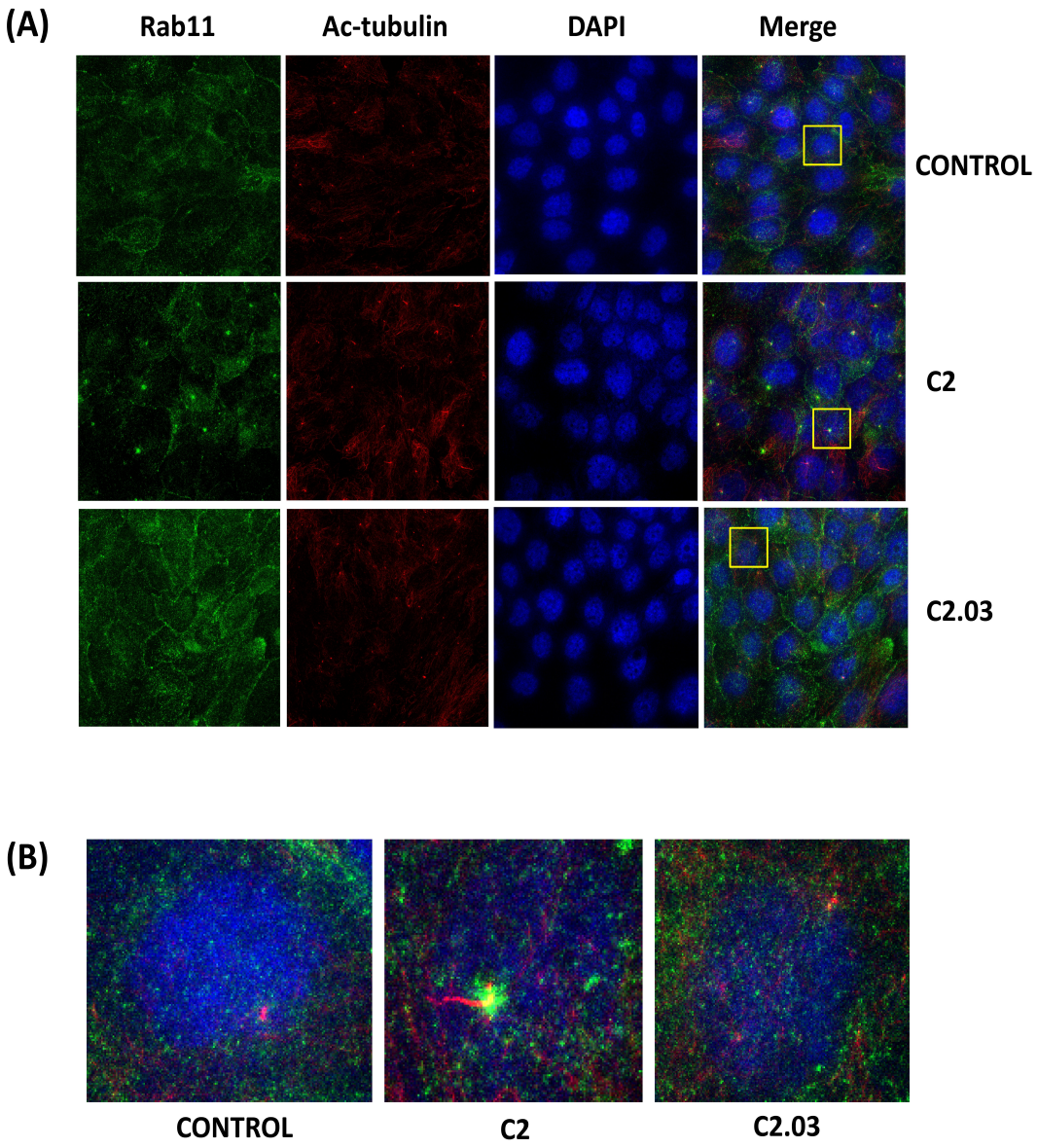

Figure 5.8. Induction of primary cilia by compound C2 is accompanied by the accumulation of Rab11 at the ciliary base. (A) Panc1 cells were treated with compound C2 or its inactive analog 2.03 for 4 days followed by staining for Rab11 (green), acetylated tubulin (red) and DAPI (blue). (B) Magnification of representative regions marked with yellow square inserts in merged images shown in (A).

5.3. Discussion

Building up on the previous work of our group that aberrant activation of SREBP1c is frequently observed in many human cancers, and that constitutively active SREBP1c suppresses the formation of the primary cilium [6], we aimed to explore and establish a new concept in the treatment of common human diseases based on reversal of SREBP1c-mediated suppression of the primary cilium. In this project we have successfully developed and established a semi-high throughput screening method based on MDCK cells that overexpress SREBP1c to mimick the lipogenic phenotype of cancer cells. We have also demonstrated the usefulness of this method as a tool to screen potent

ciliogenic modulators. Using this strategy we have identified 8 novel compounds that are cilia enhancers. These compounds have the ability to restore ciliogenesis in several cancer cell line models and in a ciliopathy model. For selected compounds we have shown that they block the growth of cancer cells in a spheroid assay in a manner associated with their ciliogenic ability, as would be expected for an inducer of ciliogenesis [15]. To what extent growth inhibition is a cause or consequence of ciliogenesis remains to be determined. This is one of the first reports on the use of pharmacological compounds to rescue cilia in cells that have lost them due to a diseased condition. These compounds might serve as potential candidates for the development of drugs not only against cancer and ciliopathies, but also against other cilium-related human diseases in general. Hence our findings open a whole new area of research and provide a framework for future development of therapies based on a novel concept of restoring cilia in diseased cells. As a future perspective, further optimization and validation of these ciliogenic compounds have to be performed by identification of their pharmacophores and their respective molecular targets. This knowledge will aid in the streamlining of mechanisms and pathways involved in the induction of ciliogenesis. However, challenges exist in further characterization of the mechanisms by which these compounds induce cilia. This is partly because of the incomplete understanding of the processes involved in ciliogenesis, which warrant further exploration. These facts together with our findings offer an interesting yet complex challenge for the future of cilia-based drug therapy.

5.4. Materials and methods

5.4.1. Cell lines

All cell lines were obtained from ATCC. MDCK cells were cultured in DMEM/F12 medium, LNCaP cells in RPMI-1640. SKBR3 cells in McCoy's 5a, WM2664, A549 and PANC-1 in DMEM, and LLC-CL4 cells in MEM-α. All media were from Life Technologies and were supplemented with 10% FBS. Fibroblasts derived from 1p36 ciliopathy patients were maintained in DMEM/F12 medium supplemented with 10% FBS. All cell lines were incubated at 37°C in a humidified incubator with 5% levels of CO_2.

5.4.2. Screening of CISTIM compound library

MDCK cells were used as a ciliogenesis model for compound screening. The cells were pre-infected with 30 p.f.u./cell of adenovirus encoding SREBP1c [10, 11] to repress

ciliogenesis followed by seeding in 96-well plates at a density of 10,000 cells/well containing 200 µl culture medium supplemented with 10% FBS. The plates were incubated at 37°C and 5% CO2 in a humidified incubator till cell confluency reached 30% after which medium was refreshed with 200ul of culture medium containing 2% FBS to reduce complexation of compound with serum proteins. The CISTIM library of 1300 compounds was provided by the Center for Innovation and Stimulation of Drug Discovery (CISTIM), (Leuven, Belgium). The compounds were stored as 10mM stock solutions in dimethyl sulfoxide (DMSO) and were added to the culture medium to a final concentration of 10µM. After 4 days, medium was replaced and fresh compound was added. After a total of 8 days of incubation, cells were chemically fixed and immunostained for cilia using an antibody against acetylated tubulin (Sigma, Cat No. T6793-.5ML). A fluorescent secondary antibody (Life Technologies, AlexaFluor, Cat No. A21145) was used against the primary antibody followed by counterstaining of nuclei with Hoechst-33258 (Cat No: 382061, Calbiochem). Images of cilia were acquired at 20X magnification in a single plane of focus using an INCell High Content Analyzer (GE Healthcare). 20 random fields were imaged per well to minimize error. Cilia were defined as structures that appeared as fluorescent spots in the image. Percentage of ciliated cells was determined using INCell Developer software by counting both cilia and nuclei. All compounds were tested in three-fold. Compounds were considered positive when they increased the percentage of ciliated cells by at least 3 standard deviations compared to vehicle (DMSO) treated cells. Toxicity/cytostatic effect was estimated by comparing nuclei count to vehicle control.

5.4.3. Confocal microscopy

The cilium modulating effect of selected compounds was confirmed by confocal fluorescence microscopy using the cell lines mentioned previously. Cells were plated on glass coverslips in 12-well plates and were grown in low serum medium (2% FCS). Treatment was started when cell cultures reached a confluency of approximately 30%. Cells were treated with the indicated compounds for 8 days with medium and compound refreshment on the 4th day. Cells were fixed in formaldehyde, permeabilized with 0.1% Triton X100, blocked with 1% BSA, incubated with 1:1000 dilution of anti-acetylated tubulin antibody (Sigma, Cat No. T6793-.5ML) for 1h followed by incubation with 1:1000 dilution of a fluorescent secondary antibody (Life Technologies, AlexaFluor, Cat. No. A21145) for 1h. Nuclei were stained with DAPI (Vector Laboratories,

Vectashield, Cat. No. H-1500). Images of primary cilia were captured by acquiring Z-stacks using a Bio-Rad confocal laser scanning microscope using a 40X oil immersion lens. Images were analyzed for cilia percentage and cilium length by ImageJ software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997-2016).

5.4.4. BrdU assay and tumor spheroid assay for cell proliferation

For WM2664 and SKBR3 cell lines, a 5-Bromo-2 deoxy-uridine Labeling and Detection Kit III (Cat No. 11444611001, Roche) was used to measure cell proliferation after treatment of the cells with compounds for 8 days.

To compare the effect of ciliogenic and inactive analogs on prostate cancer cell proliferation, tumor spheroids were formed on agarose-coated (1%) 96-well plates by seeding LNCaP prostate cancer cells at a density of 5000 cells in 200ul per well of culture medium. The plate was then kept undisturbed in an incubator at 37°C and 5% CO_2 for 4 days to facilitate spheroid formation. Treatment with compounds was started when the cells aggregated to form spheroids. Compounds and 50% of the medium were refreshed every 4th day. Images were captured at the start of treatment with 5X objective mounted on an inverted light microscope. Imaging was done every 4 days for a period of 12 days from the start of treatment. The images were analyzed by ImageJ software to calculate the differences in the volume of spheroids at different time points.

5.4.5. siRNA-mediated knockdown assay

In order to explore the ability of compounds to rescue cilia in a ciliopathy model, we performed a siRNA-mediated knockdown of the Rer1p gene in LLC-CL4 cells [12] followed by treatment with compounds or DMSO vehicle. The cells were transfected prior to seeding (reverse transfection) using lipofectamine (Thermo Fisher Scientific). Compound treatment was started 24 hours after seeding and continued for a period of 2 days. Following treatment, cells were fixed and stained for confocal microscopy to visualize cilia. Changes in cilium length were measured by Image J software.

5.4.6. Structure Activity Relationship (SAR) studies

To test the structure-function relation of novel compounds, analogs of selected positive compounds were obtained from CD3/CISTIM. Activity of compounds was tested in the

fluorescence microscopy-based ciliogenesis assay in 96-well plate format as described above.

5.4.7. Statistical analysis

Statistical analysis was done using GraphPad Prism version 6 for Mac OS X (GraphPad Software, San Diego, California, USA). Data are expressed as mean ± SEM. Student's t-test was used to assess differences between two groups. Values of p less than < 0.05 were considered to be statistically significant.

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CHAPTER 6

General Discussion

General Discussion

Primary cilia are elongated microtubule-based organelles present on the external surface of most mammalian cells. They serve as antenna of the cell to detect extracellular signals and translate them into intracellular pathways that regulate cell development and homeostasis. The importance of cilia has been clearly demonstrated by a connection between cilia and a group of numerous diseases termed as ciliopathies. Mounting evidence from recent years points towards a compelling link between cilia and cancers. However, the exact role of the cilium in tumorigenesis remains debatable and open to exploration. A general agreement exists that the cilium provides a natural braking mechanism to control cell division because it shares the same molecular machinery that is required for cell division. Loss of the cilium thus releases the brake mechanism leading to unrestricted cell division. On the other hand, several reports provide evidence that some types of cancers are dependent on the presence of cilia for their survival and growth. Therefore, primary cilia can either inhibit or facilitate tumorigenesis depending on the cancer type. Hence, either re-expression or inhibition of the cilium offers an attractive and novel therapeutic approach to treat a wide variety of cancers. Unfortunately, only few small molecule cilium regulators are known so far. Several screens of compounds have been reported, several of which using flagellated algae as an experimental model [1-3]. In this research project, we aimed to identify small molecule cilium modulators using human cancer cells or other mammalian cell models to screen several libraries of pharmacological and small molecule compounds to identify and test modulators of ciliogenesis for their therapeutic effect on different cancer cell line models and in a ciliopathy model.

To begin with, we aimed to identify compounds that could re-express primary cilia in cancer cells. Our immediate goal in chapter 2 was to develop and establish a screening method based on cancer cells in which the primary cilium is lost. This was achieved by using pancreatic cancer cells CFPAC-1 as a platform to develop a high content (IN Cell) analysis and semi-automated immunofluorescence microscopy-based screening method that identifies the presence of the cilium based on antibody staining of the ciliary component acetylated tubulin. This cell line was selected because of its relatively low ciliation percentage, its ability to grow as a flat monolayer and the clinical need for novel therapeutic agents for pancreatic cancer. Using this method we screened a library of

1600 commonly known drugs to identify inducers of ciliogenesis. Through this screening we showed that a diverse set of drugs are capable of inducing primary cilia in this and other cancer cell models including breast cancer and lung cancer. Classification based on their putative targets revealed ciliogenic activity of modulators of steroid receptors, neurotransmitters, ion channels, microtubules, tyrosine kinase receptors etc. We also showed that re-expression of cilia through these drugs hampered the proliferative potential of cancer cells and causes cell cycle arrest in G0-G1 phase, which is known to promote ciliogenesis. Although for certain drugs it is likely that increased ciliogenesis is a consequence of cell cycle arrest, for others we have shown that the reduction in proliferation was mediated at least in part by the expression of the cilium, as demonstrated by abolition of the cilium by chloral hydrate, which restored the proliferative potential of drug-treated cancer cells. Our findings reveal hitherto unknown activities of old compounds and fit in the concept of drug repurposing, a strategy that redirects existing drugs for a new indication or purpose, thereby decreasing the time-frame and costs of making a drug available in the market.

In chapter 3, we built on the newly identified compounds described in Chapter 2 and explored whether there are any common underlying mechanisms through which the diverse ciliogenic drugs might induce cilia in cancer cells. Since several ciliogenic drugs from our screen have been reported to release ATP from cells and because of the availability of expertise in our research consortium to investigate ATP-related effects, we aimed to investigate to what extent these ciliogenic drugs secrete ATP from cells and whether the secreted ATP is involved in the promotion of ciliogenesis. Using cultures of pancreatic cancer cells as experimental model we have experimentally shown that most of these drugs induce secretion of ATP from these cells, and that this secretion positively correlates with their ciliogenic ability. Using a panel of chemical inhibitors against different ATP-secretion pathways we showed that ATP is mainly released through the pannexin channels, although passive release by a small fraction of dying cells cannot be excluded. Treatment with the ATP-degrading enzyme Apyrase suppressed ciliogenesis in drug-treated cells, corroborating the importance of the released ATP in the ciliogenic activity of the selected drugs. Ciliogenesis was suppressed when drug-treated cells were exposed to Suramin, providing evidence for the involvement of purinergic receptor signaling in this process. Also, it has been shown previously that extracellular ATP can modulate ciliary beat frequency (CBF) through activation of purinergic receptors

present on the cilium [4-6]. Our findings point to the existence of a hitherto unknown autocrine/paracrine loop involving the extracellular ATP-purinergic signaling pathway in the induction of ciliogenesis at least by a selection of the drugs identified in Chapter 2. Several other mechanisms may be involved in parallel or may be exploited by some of the other drugs. These include changes in cAMP, intracellular calcium and others as discussed in chapter 2 and as summarized in figure 2.8. Also changes in intracellular trafficking are likely to play a key role as shown in Chapter 5. To what extent these mechanisms are interlinked and vary in different cell types remains to be investigated. In any case, the ATP-purinergic signaling loop that we identified here should be taken into consideration in future therapeutic approaches targeting the restoration of primary cilium in specific disease contexts.

Using the same screening platform as described in Chapter 2, we screened a library of 160 well-characterized kinase inhibitors (Chapter 4). Our choice of selecting a library of kinase inhibitors was influenced by reports in literature that a number of kinases are involved in ciliogenesis and in view of the fact that kinases are known to play a vital role in oncogenesis [7-11]. Interestingly this led to the identification of cilium enhancers as well as cilium suppressors. This finding is of particular interest in view of the ambiguous role of cilia in cancer, depending on the cancer type. In fact, although the cilium is thought to put a break on cancer cell proliferation and apparently tends to get lost in many tumor types including pancreas, breast and prostate cancer, it has been reported that cancers like basal cell carcinoma (BCC), medulloblastoma and a subset of glioblastomas are dependent on primary cilia for their growth and progression. This is most likely related to the dependency of these cell lines on Hedgehog signaling, which (unless constitutively activated through mutations in downstream actors such as Gli transcription factors) is dependent on the primary cilium [12]. Hence we tested two of the most potent suppressors of ciliogenesis, Herbimycin A and Kenpaullone, on cilium-dependent cancer models such as the glioblastoma cell line GL2664, which is well ciliated in the basal condition. Both the compounds were able to potently suppress ciliogenesis in this model and reduced their proliferative potential. Conversely, we tested the most potent cilium enhancers, EGFR/ErbB-2/ErbB-4 Inhibitor and Src Kinase Inhibitor I on poorly ciliated pancreatic cancer cells and showed that they enhanced ciliogenesis and also attenuated their proliferation. With respect to the mechanisms involved in the suppression of the cilium, and based on the knowledge of the molecular

targets of Herbimycin A and Kenpaullone, it can be speculated that microtubule stabilizing proteins and GSK-3β-mediated pathways are involved in the observed effects on the cilium. The cilium enhancer EGFR/ErbB-2/ErbB-4 Inhibitor may exert its ciliogenic effect by negative regulation of MAPK signaling and by regulating calcium influx into the cell. Src Kinase Inhibitor I likely maintains ciliogenesis by inhibiting NEDD9-mediated HDAC6 disassembly of the cilium. Also, it promotes the ciliogenic activity of MIM (Missing-in-Metastasis) protein, which is localized to the basal body. To what extent also here secreted ATP and/or changes in intracellular trafficking are involved remains to be investigated. Also further research is needed to delineate the role of ciliogenesis in the antineoplastic effects of the kinase inhibitors.

In the last part of this thesis project (Chapter 5) we pursued the more ambitious plan of searching for novel small molecule compounds that can induce ciliogenesis. Based on our previous findings that aberrant activation of the lipogenic transcription factor SREBP1 contributes to the suppression of the cilium in cancer cells, we designed a new screening method based on SREBP1-mediated forced suppression of primary cilia in normal MDCK kidney cells to mimic the lipogenic cancer phenotype. We applied this strategy to screen a library of 1300 small molecules to identify compounds that are capable of reversing SREBP1-mediated cilium suppression. From our screen we identified 8 such compounds, whose chemical structures cannot be revealed for intellectual property reasons. We confirmed that these compounds effectively induce cilia in a panel of lipogenic cancer cell lines. Interestingly, in a Rer1p gene knockdown model of 1p36 ciliopathy, some of our compounds had the ability to normalize the cilium length to that of unaffected healthy cells. Hence, future studies should also focus on identifying the effect of ciliary length on the severity of the disease. In our quest to identify more potent analogs of the selected ciliogenic compounds and to gain insight in the structure-activity relationship (SAR) of these compounds we compared the ciliogenic potential of the parent compounds with their respective analogs. This study revealed both more active as well as less active analogs that we used as pairs in a comparative study of their effect on the proliferation of lipogenic prostate cancer cells and their capability to restore ciliogenesis, also in the previously described ciliopathy model. Such studies may reveal important clues and insights regarding the pharmacophores responsible for ciliogenesis, which can aid in pinpointing the mechanisms through which these compounds exert their cilia-inducing effects.

Knowledge of essential and less important moieties of these molecules will also help identify potential sites for addition of labels that can help in the identification of the targets to which these molecules bind, which is one of the key questions that remain to be addressed. Using such pairs we have also provided evidence that at least some of these novel ciliogenic compounds restore ciliogenesis by normalizing the intracellular trafficking of Rab11-positive vesicles towards the base of the cilium, a process that is distorted by cancer cells by the action of SREBP1 [13]. To what extent also other mechanisms as those referred to in other chapters of this thesis manuscript are involved remains to be explored.

Overall, our results reveal that many small molecule compounds affect the expression of the primary cilium. These include many commonly used drugs, such as glucocorticoids, ion channel modulators and kinase inhibitors, revealing hitherto unrecognized actions of these compounds. To what extent these cilio-modulatory effects contribute to the therapeutic actions or even side effects of these compounds remains to be determined and represents an interesting area of further investigation. We also succeeded in identifying several new ciliogenic compounds that have the ability to normalize cilia both in cancer cells and ciliopathy models. These findings open up a new research avenue with potentially important therapeutic applications. Future efforts will have to be directed towards the identification of the structural elements that are responsible for the cilio-modulatory effects of these compounds and to identify the molecular targets and important players in the complex web of molecular interactions that regulate ciliogenic pathways. Further optimization and validation studies in vivo will be necessary to firmly establish the concept of cilium-targeting drug therapies. Although the generation of accurate *in vivo* models to study cilia-related disorders is a challenge, in the last few years several reliable *in vivo* models to study the relationship between cilium biology and disease have become available. The choice of the appropriate model depends on the type of ciliopathy or study. For instance, to investigate the therapeutic potential of ciliogenic compounds against ciliopathies arising from defects in ciliary motility (for e.g. PCD), ciliated unicellular organisms like trypanosoma serve as efficient models. To test compounds against more complex diseases that display more diverse clinical manifestations (for e.g. MKS, BBS), multicellular organisms like mouse, zebrafish, xenopus etc. are more attractive models because of their similarity with humans in terms of morphology, physiology and genetics. Zebrafish and xenopus have unique

features such as ease of breeding, transparency and well-defined developmental changes related to cilium defects that make these models particularly interesting in this context. Recently, mutant mouse models have been developed against several notable ciliopathies like MKS, BBS and others [14]. Mouse models with tagged cililary components that facilitate the visualization of cilia have also become available and can be crossed with specific cancer models [15]. These organisms are ideal models to study the *in vivo* effect of the ciliogenic compounds that we have identified in this project. Depending on the disease-specific mechanism of cilium distortion, different small molecules may be most suitable and fine-tuning of the treatment regimens will be important to limit effects on healthy cells. In view of the importance of cilia in many physiological processes and pathologies, having small molecules available that have the ability to normalize or otherwise modulate cilia might find important applications in clinical practice. The future will tell.

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Summary

In this doctoral thesis project we have aimed to identify small molecules that modulate the formation of the primary cilium. This is a microtubule-based cellular antenna that protrudes from many mammalian and human cells and that is distorted in many diseases, including so-called ciliopathies but also in more common conditions such as cancer. To this end we have developed a microscopy-based semi-high throughput screening method and have used it to screen three chemical libraries: a repurposing library of FDA-approved drugs, a library of kinase inhibitors and a library of novel compounds. Altogether we have identified more than 100 known and novel compounds that act either as enhancers or suppressors of ciliogenesis. Among the identified compounds are commonly used drugs including glucocorticoids, ion channel modulators and kinase inhibitors. Several of these compounds have the ability to normalize or modulate the primary cilium in various cancer models and to attenuate cancer cell proliferation. Some of these compounds also normalize the cilium in a ciliopathy model. These compounds may act through multiple mechanisms including the secreted ATP-purinergic signaling axis and the modulation of intracellular vesicle trafficking. These findings are among the first on the identification and use of pharmacological compounds to modulate cilia in cells in a diseased context and provide a framework for the future development of novel cilium-targeting therapies for cancer and ciliopathies.

Samenvatting

In de hier voorliggende doctoraatsthesis hebben we tot doel gesteld om chemische moleculen te identificeren die de vorming moduleren van het primaire cilium. Dit is een microtubule-gebaseerde cellulaire antenne die uitsteekt vanaf het oppervlak van vele zoogdier- en humane cellen en die verstoord is in heel wat aandoeningen gekend als ciliopathieën, maar ook in meer voorkomende ziektes zoals kanker. Hiertoe hebben we een op microscopie gebaseerde semi-hoge doorvoer screeningsmethode ontwikkeld, waarmee we drie chemische bibliotheken gescreend hebben: een 'repurposing' bibliotheek van FDA-erkende geneesmiddelen, een bibliotheek van kinase inhibitoren en een bibliotheek van nieuwe chemische stoffen. Alles tezamen hebben we meer dan 100 gekende en nieuwe chemische moleculen geïdentificeerd die werken als promotoren of inhibitoren van de ciliogenese. Onder de geïdentificeerde moleculen bevinden zich veelgebruikte geneesmiddelen zoals glucocorticoïden, modulatoren van ionenkanalen en kinase-inhibitoren. Verscheidene van deze moleculen zijn in staat het primaire cilium te normaliseren of te moduleren in verschillende kankermodellen en hun celproliferatie te remmen. Sommige van deze stoffen nomaliseren ook het cilium in een ciliopathiemodel. Deze moleculen werken via verschillende mechanismen, waaronder de gesecreteerde ATP-purinergische signaaltransductieweg en de modulatie van het intracellulaire vesikelverkeer. Deze bevindingen zijn één van de eerste wat betreft de identificatie en het gebruik van farmacologische middelen om cilia te moduleren in cellen in een ziektetoestand en vormen het kader voor de toekomstige ontwikkeling van nieuwe op het cilium gerichte therapieën voor kanker en ciliopathieën.

CURRICULUM VITAE

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SUMMARY

11 years of research and development (R&D) experience in the biotech industry and academia with a proven record of successful handling of complex and diverse projects.

WORK EXPERIENCE

- May 2010 – September 2016: Doctoral researcher in the Laboratory of Lipid Metabolism and Cancer, Dept. of Oncology, Faculty of Medicine, KU Leuven, Belgium.

- December 2009 - May 2010: Doctoral researcher in the Laboratory of Experimental Radiotherapy, Dept. of Oncology, Faculty of Medicine, KU Leuven, Belgium.

- August 2005 - November 2009: Senior Research Associate in the R&D department of Shantha Biotech (Hyderabad, India), a member of Sanofi Pasteur, France.

TECHNICAL SKILLS

- Biomarker and small molecule drug discovery for cancers.
- High-throughput screening of chemical compound libraries.
- Construction and serological screening of cDNA libraries.
- SAR (Structure-Activity Relationship) assays and studies.
- Drug dose response assays.
- Development and optimization of cell-based functional assays.
- Cell culture, molecular biology and microbiology techniques.
- siRNA knock-down assays.
- Immunofluorescence techniques and confocal microscopy.
- Production of anti-cancer monoclonal antibodies from transfected mammalian CHO cell-lines by suspension cell fermentation technology.
- High Density Fermentation and Downstream Processing of genetically engineered Pichia pastoris and E.coli designed to produce therapeutic recombinant proteins.
- Pilot scale production of capsular polysaccharides by fermentation of Streptococcus pneumoniae in bioreactors.

- Process development of vaccines against infectious bacterial and viral diseases.
- Direct and Indirect ELISA tests (to measure antigen content in vaccine test samples / IgG antibody concentration in serum samples).

PROJECTS HANDLED

In the Laboratory of Lipid Metabolism and Cancer, Department of Oncology, KU Leuven:

- Screened a repurposing library of 1600 FDA-approved drugs. This work has led to the development and optimization of a screening method to identify cilia-inducing compounds. Using this method, different classes of cilia inducers have been identified which might serve as potential drugs against pancreatic and other types of cancers. This research has been published in the journal 'Oncotarget'.

- Screened a library of 4500 small molecules. This led to the identification of 8 novel ciliogenic compounds and several cilia inhibitors, which are potential candidates for the development of cancer therapeutics.

- Screened a library of 160 kinase inhibitors to study the effect on cilia induction in cancer cells. This work has led to the identification of several compounds that are effective against glioblastomas.

In the R&D department of Shantha Biotech, Hyderabad, India:

- Worked on Oral Cancer Project, which involved construction of oral tumor cDNA library and serological screening for novel antigens by SEREX technology. This work resulted in the identification of several proteins that are being evaluated for their utility as biomarkers to develop a diagnostic method for oral cancer.

- Worked on the Process Development of a multivalent pneumococcal capsular polysaccharide conjugate vaccine. This work involved the designing of experiments to isolate the antigenic polysaccharides from the capsules of Streptococcus pneumoniae and to conjugate toxoids to the isolated polysaccharides.

- Produced therapeutic recombinant proteins HBsAg (Hepatitis B surface antigen), HPV16L1 (Human Papilloma Virus Antigen), tPA (tissue Plasminogen Activator), GCSF (Granulocyte Colony Stimulating Factor), by fermentation of genetically engineered bacteria and yeast in fermenters on a pilot scale.

- Involved in the production of anti-cancer monoclonal antibodies from transfected CHO cell lines by fermentation technology.

EDUCATIONAL QUALIFICATIONS

- Ph.D. in Biomedical Sciences, KU Leuven, Leuven, Belgium (2016).
- M.Sc. in Bioinformatics, Annamalai University, Tamil Nadu, India (2005)
- M.Sc. in Microbiology, Sri Krishnadevaraya University, A.P., India (2003).
- B.Sc. in Microbiology, Sri Krishnadevaraya University, A.P., India (2001).

PUBLICATIONS

- Identification of drugs that restore primary cilium expression in cancer cells - **Khan NA**, Willemarck N, Talebi A, Marchand A, Binda MM, Dehairs J, Rueda-Rincon N, Daniels VW, Bagadi M, Raj DB, Vanderhoydonc F, Munck S, Chaltin P and Swinnen JV. (Oncotarget, February 2016).
- Primary cilium suppression by SREBP1c involves distortion of vesicular trafficking by PLA2G3 - Gijs HL, Willemarck N, Vanderhoydonc F, **Khan NA**, Dehairs J, Derua R, Waelkens E, Taketomi Y, Murakami M, Agostinis P, Annaert W, Swinnen JV. (Molecular Biology of the Cell, June 2015).
- p53 attenuates AKT signaling by modulating membrane phospholipid composition - Rueda-Rincon N, Bloch K, Derua R, Vyas R, Harms A, Hankemeier T, **Khan NA**, Dehairs J, Bagadi M, Binda MM, Waelkens E, Marine JC, Swinnen JV. (Oncotarget, August 2015).
- Drug-induced ciliogenesis in pancreatic cancer cells is facilitated by the secreted ATP-purinergic receptor signaling pathway - **Khan NA**, Garg AD, Agostinis P, Swinnen JV. (Submitted).

POSTERS PRESENTED

- 'p53 attenuates AKT signaling by modulating membrane phospholipid composition' presented at VIB conference "Metabolism in Cancer and Stromal Cells" held in Leuven (Belgium) in September 2015.
- 'Identification of Ciliogenic Compounds using Pancreatic Cancer Cells as a Screening Model' presented at "Cilia 2014" conference held at Institute Louis Pasteur, Paris (France) in November 2014.
- 'p53 regulates Akt/PKB signaling through stearoyl-CoA desaturase (SCD1)-mediated changes in membrane phospholipids' presented at Keystone Symposia "Tumor Metabolism" held in British Columbia (Canada) in March 2014.
- 'Modulation of cancer membrane phospholipid composition by p53' presented at the conference "Cancer and Metabolism 2013" held in Amsterdam (Netherlands) in June 2013.

- 'Superparamagnetic nanoparticles for targeted plasma membrane OMICS' presented at "Beatson International Cancer Conference - Membrane Dynamics in Cancer 2012" held in Glasgow, Scotland (UK) in July 2012.

CONFERENCES ATTENDED

- "Cilia 2014" organized at Institute Louis Pasteur, Paris, France in November 2014.

- "Oncoforum 2013" organized by Leuven Cancer Institute (LKI) and Doctoral School of Cancer, KU Leuven, Leuven, Belgium in May 2013.

- "Cilia 2012" organized by Ciliopathy Alliance at the Institute of Child Health, UCL, London, UK in May 2012.

- "Entrepreneurship in Life Sciences" workshop organized by Flanders Institute of Biotechnology (VIB) and Vrije Universiteit Brussel (VUB) at Brussels in May 2012.

MEMBERSHIPS AND AWARDS

- Life Member of Association of Microbiologists of India (AMI).

- 2009 -2011: KU Leuven Ph.D. scholarship.